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Small circular DNA of *Drosophila melanogaster*: Chromosomal homology and kinetic complexity

(middle repetitive DNA/translocatable elements)

SHARON W. STANFIELD*† AND JUDITH A. LENGYEL*‡

*Biology Department and Molecular Biology Institute, University of California, Los Angeles, California 90024

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ABSTRACT **Nucleic acid reassociation techniques were used to determine the kinetic complexity of small circular DNA in cultured cells of *Drosophila melanogaster*. Two kinetic components are present. One of these constitutes 82% of the mass of the circular DNA and has a complexity of 1.8×10^4 nucleotide pairs; the other constitutes 18% of the mass and has a significantly higher but undefined sequence complexity. We have demonstrated that these circular molecules hybridize to middle repetitive chromosomal sequences by hybridization of *in vitro*-labeled circular DNA tracer with a vast excess of *Drosophila* chromosomal DNA. Thermal stability measurements indicate that base-pair mismatch between small circular DNA and middle repetitive chromosomal DNA does not exceed 2%. We discuss possible functions of these small circular DNAs in light of the above findings.**

Heterogeneous closed circular DNA is an intriguing and little understood class of molecules that has been described in a wide variety of eukaryotic organisms, including *Neurospora, Euglena*, trypanosomes, yeast, tobacco, *Xenopus*, and boar, and in cell culture lines from monkey, mouse, and human (1–9).

We have investigated this class of molecules in embryos and cultured cells of *Drosophila melanogaster* (10) because of the unique genetic and cytological advantages that this organism offers for the study of structure and function of DNA sequences. In the cultured cells (Schneider's line 2), small circular DNA is predominantly nuclear and exhibits a buoyant density in neutral CsCl indistinguishable from that of the main band nuclear DNA. The circular molecules range in size from approximately 300 to >7500 nucleotide pairs (Nt Pr) with an average size of 3300 Nt Pr. The size distribution and average circle size of small circular DNA in embryos and in a cloned subline of Schneider's line 2 cells differ significantly from that of Schneider's line 2. Both logarithmic and stationary phase cells contain a minimum of 3 to a maximum of 40 average-sized small circular DNA molecules per cell, constituting a maximum of 0.03% of the total cellular DNA (10).

Although the function of heterogeneous circular DNA is unknown, it has been suggested that it might be involved in various genetic regulatory phenomena in *Drosophila* via integration into and excision from chromosomal DNA (11–17). Because an understanding of the function of these circular molecules depends upon the characterization of the sequences contained within them, we have determined the sequence complexity of *Drosophila* heterogeneous circular DNA and its homology to chromosomal DNA. Our finding that these circles are complementary to middle repetitive sequences of the *Drosophila* genome supports the notion that circles may interact with chromosomal DNA.

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MATERIALS AND METHODS

Purification and *In Vitro* Labeling of Small Circular DNA. Covalently closed circular DNA was prepared from cleared lysates of Schneider's line 2 cells (18) by a modification of the procedure of Stanfield and Helinski (10). After purification in three consecutive CsCl/ethidium bromide gradients, circular DNA was digested with RNase T1 (20 μg/ml), α-amylase (100 μg/ml), and preboiled RNase A (20 μg/ml) in 15 mM NaCl/1.5 mM Na citrate, pH 7.2/0.02 M Na_2 EDTA for 30 min at 37°C. Sarkosyl was added to 1% and the DNA was recentrifuged on two consecutive analytical grade CsCl gradients to separate mitochondrial (ρ = 1.680 g/ml) from small circular (ρ = 1.703 g/ml) DNA. Small circular DNA was quantitated by electron microscopy, using added plasmid R6K as an internal standard (10).

Small circular DNA was labeled *in vitro* with ^3H or ^{32}P to specific activities of 1.3×10^6 cpm/μg or 1.8×10^8 cpm/μg, respectively, by using the "nick translation" procedure of Rigby *et al.* (19). The small circular DNA tracer preparation used in the chromosome homology experiment (Fig. 2) was significantly contaminated with mitochondrial DNA when originally prepared. This was apparently due to an increase in the weight ratio of mitochondrial to small circular DNA that occurred in the cultured cells during the course of these experiments. Because small circular DNA does not hybridize to mitochondrial DNA (see legend of Fig. 1), we removed more than 98% of contaminating mitochondrial DNA from the tracer by hybridization to a large excess of highly purified unlabeled mitochondrial DNA, followed by hydroxylapatite (HAP) chromatography. The final preparation was contaminated by 12% reactable mitochondrial DNA as determined by hybridization to excess unlabeled mitochondrial DNA.

Preparation of Unlabeled Chromosomal DNA. Unlabeled chromosomal DNA was prepared by two different procedures. (*i*) *Isolation from purified nuclei.* Pelleted nuclei from a 2% Triton X-100 lysate were washed in 0.05 M Tris·HCl, pH 8.0/0.03 M Na_2EDTA/7.35% (wt/vol) sucrose and resuspended in TES buffer (0.03 M Tris·HCl, pH 8.0/0.05 M NaCl/5 mM Na_2EDTA). Sarkosyl was added to 1% and the viscous lysate was centrifuged to equilibrium in a CsCl gradient. The viscous band was pooled and dialyzed against TES. Supercoiled Col E1 DNA (6600 Nt Pr) marker was added and 250-μg portions of the chromosomal DNA were sedimented through 15–50%

Abbreviations: Nt, nucleotide(s); Nt Pr, nucleotide pairs; C_0t, initial concentration of DNA (moles of nucleotide/liter) × time (seconds); EC_0t, equivalent DNA conditions; PERT, phenol emulsion reassociation technique; HAP, hydroxyapatite; t_m, melting temperature (of double-stranded nucleic acid).

† Present Address: Biology Department, B-022, University of California at San Diego, La Jolla, CA 92093.
‡ To whom reprint requests should be addressed.

neutral sucrose gradients at 18,000 rpm for 16 hr at 16°C in an SW27 rotor. Rapidly sedimenting fractions (>33 S) containing less than 1% of the supercoiled Col E1 DNA were pooled and digested with RNase A, RNase T1, and α-amylase as described above for the purification of circular DNA. Pronase (1 mg/ml, predigested for 30 min at 60°C) was added, incubation was continued for 30 min, and the reaction was then terminated with 1% Sarkosyl. The DNA was centrifuged to equilibrium in neutral CsCl, pooled, sonicated, and passed over Sephadex SP-C25 and Chelex.

(*ii*) *Isolation from pellets of cleared lysates of whole cells.* Pellets were slowly resuspended in TES during extended digestion with proteinase K at 4°C. Large DNA was then pelleted and resuspended twice by using the procedure described by Hirt (20). The final pellet was resuspended in distilled water, brought to 0.02 M NaCl, sonicated, and purified by HAP chromatography in 8 M urea (21).

DNA/DNA Reassociation by the Standard Aqueous Technique. All DNAs were sonicated (except for *in vitro*-labeled DNA) and then extracted with chloroform and passed over Sephadex SP-C25 and Chelex. DNA reaction mixtures were denatured at 110°C and incubated in 0.48 M sodium phosphate, pH 6.8/0.06% sodium dodecyl sulfate at 70°C, and reactions were terminated by freezing in liquid nitrogen. Frozen samples were thawed rapidly, diluted into 0.14 M sodium phosphate, pH 6.8/0.2% sodium dodecyl sulfate, and passed over HAP at 56.5°C to separate double-stranded from single-stranded molecules (22). Column effluents were assayed for radioactivity in Aquasol-2. Zero time values were obtained by denaturing the DNA in 0.14 M sodium phosphate, chilling immediately in ice water, and assaying on HAP. C_0t values were corrected to equivalent DNA (EC_0t) conditions (60°C, 0.12 M phosphate buffer) by multiplying by 5.65 (23).

DNA/DNA Reassociation by the Phenol Emulsion Reassociation Technique. The phenol emulsion reassociation technique (PERT) of Kohne *et al.* (24) was used to reassociate very low concentrations (1–2 ng/ml) of *in vitro*-labeled small circular DNA. Denatured small circular DNA was shaken at room temperature on a Vortex Genie mixer, at one-half maximal speed, in 0.13 M sodium phosphate, pH 6.8/1.67 M sodium perchlorate/9% phenol. Reactions were terminated by diluting aliquots into 0.14 M sodium phosphate, pH 6.8/0.2% sodium dodecyl sulfate followed by chromatography on HAP. The PERT C_0t values were corrected to EC_0t by multiplying by 3×10^4.

RESULTS

Kinetic Complexity of Small Circular DNA. The size heterogeneity of the small circular DNA (0.3–2.1 × 10^4 Nt Pr) raises the question of the total sequence complexity contained within the population of circles. Previous data ruled out the possibility that small circular DNA consisted of repeats of a single short sequence (10), but no estimates of total sequence complexity were made due to the difficulty of obtaining sufficient material to generate high C_0t values under standard aqueous conditions. By using the PERT (24), which greatly increases the rate of DNA/DNA reassociation, we were able to study the self-hybridization of small circular DNA with the small amount of material available. The PERT conditions used here increase the rate of hybridization for *Escherichia coli* DNA approximately 20,000- to 40,000-fold over that observed under standard aqueous (EC_0t) conditions.

The self-annealing of small circular DNA in Fig. 1 shows that data obtained in an aqueous hybridization system overlap with data obtained with the PERT when the latter are multiplied by 3×10^4. Duplicate reassociations of small circular DNA by

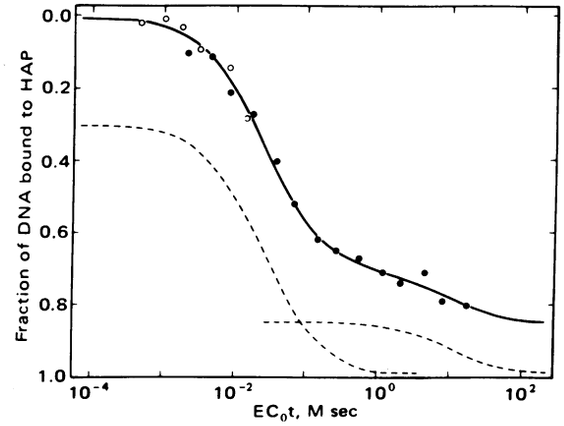

FIG. 1. Self-reassociation of small circular DNA. Small circular [^{3}H]DNA, 480 nucleotides (Nt) in length, was reassociated by using both the PERT (1.4 ng/ml)(●) and the standard aqueous technique (4.2 ng/ml)(○). The small circular DNA preparation was contaminated with less than 5% reactable mitochondrial DNA and less than 2% chromosomal DNA. The amount of contaminating mitochondrial DNA was determined by hybridization of a portion of the nick-translated circular [^{3}H]DNA preparation to an excess of purified mitochondrial [^{14}C]DNA. The amount of contaminating chromosomal (linear) DNA was determined by electron microscopy prior to *in vitro* labeling. The solid line through the data points represents the best least-squares solution, assuming two second-order kinetic components (25). The dashed curves represent the elements of the overall solution.

using the PERT, performed in two different laboratories with two different preparations of small circular DNA, yielded rate constants that differed by no more than a factor of 2. This supports our belief that the PERT yields reasonably accurate kinetic complexity data for the bulk of small circular DNA. By using the PERT, we find that *Drosophila* small circular DNA reassociates as two components whose observed rate constants (k_{obs}) differ by over 2 orders of magnitude (see Table 1). At the highest C_0t values obtained, 85% of the DNA reacted; the remaining nonhybridizing DNA probably consists of very short sequences created by the *in vitro* labeling procedure. The major, rapidly renaturing component thus constitutes 82%, and the minor, slowly renaturing component constitutes 18% of the total reactable small circular DNA.

The complexity of the major component of small circular DNA was calculated by using our determination of the rate constant for single-copy *Drosophila* chromosomal DNA under aqueous conditions (Fig. 2) as a kinetic standard. The rate constants of pure single copy chromosomal DNA and pure major component small circular DNA are 0.012 M^{-1} sec^{-1} and 68 M^{-1} sec^{-1}, respectively [corrected to the rate expected for 690-Nt Pr DNA (see Table 1)]. From the ratio of these rate constants and the complexity of *Drosophila* single copy DNA [$0.56 \times (1.8 \times 10^8$ Nt Pr) = 1.0×10^8 Nt Pr, calculated from Table 1 and ref. 28], we calculate that the major component of small circular DNA has a complexity of 1.8×10^4 Nt Pr.

The rate constant for the pure minor component of small circular DNA seen in Fig. 1 is 0.74 M^{-1} sec^{-1} (Table 1). Because this slower reacting component represents a minor fraction of the total small circular DNA, and could be due in part to the presence of short or damaged sequences in the *in vitro*-labeled preparation, we prefer not to make an estimate of its complexity other than to say that it is significantly more complex than the 1.8×10^4-Nt Pr component.

Homology of Small Circular DNA to Chromosomal DNA. In order to study possible homology between small circular DNA and chromosomal DNA, we performed hybridization

Table 1. Kinetic components of small circular DNA and chromosomal DNA

Component	$F1$*	$F2$†	k_{obs}, M^{-1} sec^{-1}	$k_{adj\ to\ 690\ Nt}$,‡ M^{-1} sec^{-1}	k_{pure},¶ M^{-1} sec^{-1}
Self-annealed small circular [^{3}H]DNA:					
Low-complexity component	0.70	0.82	40	48	68
High-complexity component	0.15	0.18	0.093	0.11	0.74
Chromosomal [^{3}H]DNA driven by unlabeled chromosomal DNA:					
Very highly repetitive and foldback	0.26	0.27	Not defined	—	—
Middle repetitive	0.16	0.17	0.43	0.50	3.1
Single copy	0.53	0.56	0.0056	0.0064	0.012
Tracer small circular [^{32}P]DNA driven by chromosomal DNA	—	—	0.12	0.74	—

Kinetic parameters were obtained by computer fit of data in Figs. 1 and 2.

* The fraction of total labeled DNA in each component.

† The fraction of total labeled reactable DNA in each component.

‡ The rate constants for the different hybridizations were corrected for disparity in tracer and driver lengths (26) and adjusted to the rate for 690-Nt DNA (27) for ease of comparison as follows: self-annealed small circular [^{3}H]DNA, $k_{adj\ to\ 690\ Nt} = k_{obs}\ (690/480)^{1/2}$; self-annealed chromosomal [^{3}H]DNA, $k_{adj\ to\ 690\ Nt} = k_{obs}\ (690/600)^{1/2}\ (690/600)^{1/2}$; small circular [^{32}P]DNA tracer driven by chromosomal DNA, $k_{adj\ to\ 690\ Nt} = k_{obs}\ (690/110)^{1/2}\ (690/110)^{1/2}$.

¶ $k_{pure} = k_{adj\ to\ 690\ Nt}/F1$.

experiments by using a vast excess of *Drosophila* chromosomal DNA and trace amounts of labeled small circular DNA. Chromosomal DNA driver was freed of greater than 99% of the contaminating small circular DNA by differential sedimentation. Therefore, under the chromosomal DNA excess conditions of the hybridization reaction shown in Fig. 2, the small circular [^{32}P]DNA can reassociate only if homologous sequences are present in the chromosomal DNA.

A large fraction of the small circular [^{32}P]DNA tracer was driven into hybrid by chromosomal DNA (Fig. 2). The kinetics of this single-component reaction yielded a corrected rate

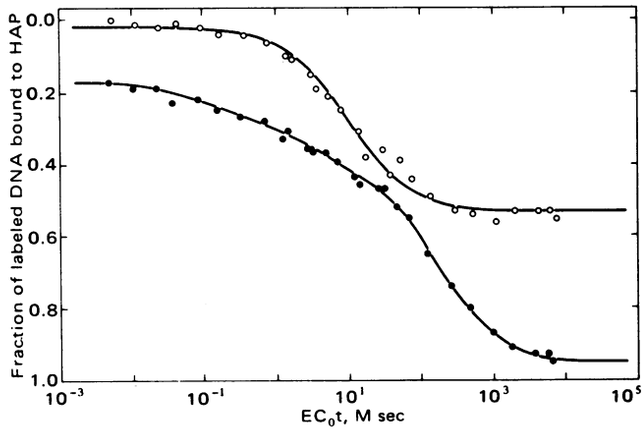

FIG. 2. Reassociation of small circular DNA with chromosomal DNA. Approximately 0.4 ng of small circular [^{32}P]DNA per ml (○) was hybridized to a 500,000-fold excess of unlabeled chromosomal DNA containing a small amount of chromosomal [^{3}H]DNA (●) by using the aqueous technique. The small circular [^{32}P]DNA preparation was contaminated by 12% reactable mitochondrial DNA. The sizes of the circular [^{32}P]DNA, the unlabeled chromosomal DNA, and the chromosomal [^{3}H]DNA, as determined by alkaline sedimentation, were 110, 690, and 600 Nt, respectively. The ratio of unlabeled DNA to chromosomal [^{3}H]DNA was 60:1. A zero-time binding of 1.5% was subtracted from each of the small circular DNA data points (○). No zero-time binding was subtracted from the chromosomal DNA data points (●). The solid curves through the data points represent the best least-squares solution for three components for chromosomal DNA and one component for circular DNA. A control experiment, in which approximately 0.4 ng of small circular [^{32}P]DNA per ml was mixed with a 500,000-fold excess of unlabeled calf thymus DNA showed 3.3% hybridization by an EC_0t of 980 M sec after a zero-time value of 1.5% was subtracted.

constant of 0.74 M^{-1} sec^{-1} (see Table 1). The ratio of this rate constant to that for single copy DNA indicates that the average sequence homologous to small circular DNA is present about 120 times per haploid genome. The moderately repetitive DNA sequences of *Drosophila* (corrected rate constant = 0.50 M^{-1} sec^{-1}, Table 1) are present about 78 times per haploid genome. Therefore, the great majority of chromosomal DNA sequences homologous to small circular DNA can be considered to be members of the moderately repetitive class. No significant hybridization of small circular DNA to either highly repetitive or single copy DNA was observed.

Because chromosomal sequences homologous to small circular DNA are 120-fold repeated and the complexity of the major component of small circular DNA is 1.8×10^4 Nt Pr, the sequences homologous to small circular DNA represent about 1.2% of total chromosomal DNA. There are thus approximately 40 times more small circular DNA-homologous sequences present in the chromosomal DNA than in the DNA fraction characterized as small circular, which constitutes a maximum of 0.03% of the total DNA (10). This conclusion is supported by the fact that the rate of reaction of small circular DNA with total unfractionated cellular DNA (data not shown) is not detectably different from its rate of reaction with chromosomal DNA that lacks the small circular DNA fraction.

Because 12% of the small circular DNA tracer consisted of contaminating mitochondrial DNA capable of hybridizing to contaminating mitochondrial DNA in the driver, we also attempted to fit the tracer data to two components. Whether the mitochondrial DNA was considered to renature either more slowly or more rapidly than the small circular DNA, however, the adjusted rate constants for the small circular DNA component (0.42 or 1.2 M^{-1} sec^{-1}) were not significantly different from that determined for moderately repetitive chromosomal DNA (0.50 M^{-1} sec^{-1}). Thus, contamination of small circular DNA by mitochondrial DNA does not alter our conclusion that a large fraction of small circular DNA is homologous to moderately repetitive chromosomal sequences.

The final level of hybridization of 55% shown in Fig. 2 probably represents the total reactability of the circular DNA used in this experiment, because the average size of the *in vitro*-labeled circular DNA was very small (110 Nt). In other experiments (not shown) in which the small circular DNA tracer was significantly larger and essentially free of contaminating mitochondrial DNA, as much as 70% of the small circular DNA

was driven into hybrid by chromosomal DNA at EC_0t values below those at which significant self-hybridization of circular DNA occurred. Because the maximum reactability of the *in vitro*-labeled circular DNAs used in these experiments never exceeded 80%, we consider it likely that small circular DNA is entirely homologous to chromosomal DNA, specifically, to the middle repetitive sequences of chromosomal DNA.

Stability of Duplexes Formed Between Small Circular and Chromosomal DNA. To determine the degree of sequence homology, we compared the melting behavior of small circular DNA with that of hybrids between small circular DNA and chromosomal DNA. Upon heating, *in vitro*-labeled native small circular DNA melted with two apparent transitions (Fig. 3*A*). Approximately 13% of the small circular DNA (probably representing very small, thermally unstable pieces) melted below 75°C. The remainder melted with a thermal denaturation temperature (t_m) of approximately 87.6°C, or about 1.6°C below that of native chromosomal DNA (t_m = 89.2°C, Fig. 3*A*). This small difference in t_m is probably due to the fact that the circular DNA used in this experiment was smaller in size than the chromosomal DNA (23). The assumption that the material in native small circular DNA that melted at the lower temperature consists of very small DNA is supported by the melt of renatured small circular DNA. In this case, only a single transition is seen with a t_m of 87.0°C (Fig. 3*B*).

Duplexes between small circular DNA and chromosomal DNA (Fig. 3*B*) melted over a broader temperature range than did self-annealed small circular DNA, with a t_m of 85.2°C. The difference of 1.8°C in t_m between self-reassociated small circular DNA and small circular DNA annealed to chromosomal DNA indicates a base pair mismatch between small circular DNA and middle repetitive chromosomal DNA sequences of approximately 2% (29).

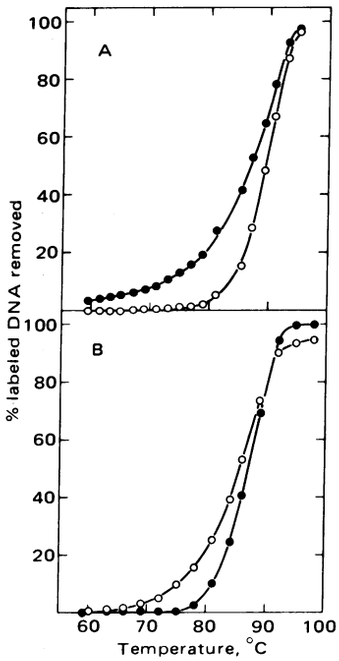

FIG. 3. Thermal dissociation profiles of native and renatured small circular DNA. (*A*) Native, *in vitro* ^{3}H-labeled small circular DNA of average size 240 Nt Pr, containing less than 4% contaminating mitochondrial DNA, (●) and native chromosomal [^{14}C]DNA (400 Nt Pr) (○) were mixed and melted together. (*B*) Small circular [^{3}H]DNA, denatured and self-hybridized to an EC_0t of 8.5 M sec (72% renatured) by using the PERT, was melted (●); small circular [^{3}H]DNA hybridized to a 20,000-fold excess of unlabeled chromosomal DNA to an EC_0t of 30 M sec (55% hybridized) was melted (○). The latter two small circular [^{3}H]DNA preparations were identical to that described in the legend of Fig. 1. The DNAs were bound to HAP at 56.5°C, then released by washing with 0.14 M phosphate buffer at 2°C temperature intervals increasing from 59°C to 98°C. Eluates were collected in scintillation vials and assayed for radioactivity in the presence of Aquasol-2. At the end of the experiment, irreversibly adsorbed DNA was detected by dissolving the HAP in 6 M HCl and assaying for radioactivity. The label in this fraction was summed with that of the other fractions to obtain total input counts. The failure of three of the curves to reach 100% was due to the irreversible binding of less than 5% of the DNA to the HAP.

DISCUSSION

Sequence Complexity of Small Circular DNA. Self-annealing experiments demonstrate that a major portion (82%) of small circular DNA has a sequence complexity of approximately 1.8×10^4 Nt Pr. A minor component, with a higher but undefined sequence complexity, is also indicated by the data. The maximal sequence complexity that could be contained in the circular DNA of an average cell is 1.3×10^5 Nt Pr, based on an average circle size of 3300 Nt Pr and a maximum of 40 circles per cell (10). Each cell could thus contain all sequences in the 1.8×10^4-Nt Pr complexity component. Whether the circular DNA is present in all cells, or is restricted to a subset of the cell population, however, remains an open question.

Homology Between Small Circular DNA and Chromosomal DNA. The major portion (possibly all) of small circular DNA hybridizes to chromosomal DNA and is homologous to middle repetitive DNA (Fig. 2). There is no detectable homology with highly repetitive (satellite) or single-copy DNA.

The nucleotide sequences in small circular DNA are of near perfect complementarity, indicated by the fact that native and self-reassociated circular DNA have nearly identical thermal stabilities (Fig. 3). There is thus very little divergence among the sequences present in the small circular DNA population. Hybrids between small circular DNA and chromosomal DNA melt at a slightly lower temperature, however, than self-reassociated small circular DNA, indicating a base pair mismatch of approximately 2%. This result would be expected, even if small circular DNA were perfectly matched with certain chromosomal sequences, because families of intermediate repeat DNA are mismatched by 3–7% in *Drosophila* (30). The mismatch of only 2% suggests that the sequences in small circular DNA belong to well-matched families of repeated sequences.

Possible Function of Small Circular DNA. The high degree of homology between small circular DNA and middle repetitive chromosomal DNA allows the speculation that small circular DNA has the capacity to insert into, and excise from, chromosomal DNA. If the circles are indeed mobile, they might be analogous to translocatable elements of prokaryotes, various representatives of which are known to cause translocation of gene sequences, to stimulate the formation of deletions, and to act as promoters or terminators of transcription (for review, see ref. 31). In eukaryotes, circular DNA with the properties of translocatable elements has been postulated to explain a number of genetic phenomena, such as the presence of "controlling elements" in maize (ref. 32; reviewed in ref. 33), high mutability and reversion at certain loci in *Drosophila* (13), the rectification of moderately repetitive chromosomal sequences (11), and the dispersion of repeated structural genes in the *Drosophila* genome (15).

If small circular DNA were altering gene activity by continually interacting with the chromosome, one might expect the population of circles to differ in cells containing different sets of active genes. Although sequence complexities have not

been compared, the size distributions of small circular DNA have been shown to differ in *Drosophila* cultured cells and embryos (10) and in bursae of chicken embryos and chicks (34).

In a previous paper (10), we speculated on the possibility that small circular DNA represents amplified moderately repetitive *Drosophila* genes, the most obvious of which is ribosomal DNA. We have found no evidence, however, for significant homology of small circular DNA to either ribosomal or transfer RNAs; the homology of small circular DNA to other cellular RNAs is the subject of another report (Stanfield and Lengyel, unpublished results).

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