Unusual properties of genomic DNA molecules spanning the euchromatic – heterochromatic junction of a *Drosophila* minichromosome

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ABSTRACT

While investigating the copy number of minichromosome *Dp(1;f)1187* sequences in the polyploid chromosomes of ovarian nurse and follicle cells of *Drosophila melanogaster* we discovered that restriction fragments spanning the euchromatic – heterochromatic junction of the chromosome and extending into peri-centromeric sequences had the unusual property of being selectively resistant to transfer out of agarose gels during Southern blotting, leading to systematic reductions in *Dp1187*-specific hybridization signals. This property originated from the peri-centromeric sequences contained on the junction fragments and was persistently associated with *Dp1187* DNA, despite attempts to ameliorate the effect by altering experimental protocols. Transfer inhibition was unlikely to be caused by an inherent physical property of repetitive DNA sequences since, in contrast to genomic DNA, cloned restriction fragments spanning the euchromatic – heterochromatic junction and containing repetitive sequences transferred normally. Finally, the degree of inhibition could be suppressed by the addition of a Y chromosome to the genotype. On the basis of these observations and the fact that peri-centromeric regions of most eukaryotic chromosomes are associated with cytologically and genetically defined heterochromatin, we propose that peri-centromeric sequences of *Dp1187* that are incorporated into heterochromatin *in vivo* retain some component of heterochromatic structure during DNA isolation, perhaps a tightly bound protein or DNA modification, which subsequently causes the unorthodox properties observed *in vitro*.

INTRODUCTION

Eukaryotic chromosomes commonly contain 'heterochromatic' regions, often peri-centromeric in location and rich in repetitive

DNA sequences, that appear cytologically condensed throughout the cell cycle (1). By contrast, euchromatic chromosomal regions decondense during interphase of the cell cycle and are rich in unique sequences. In addition to cytological appearance, a number of genetic phenomena are used, in part, to define a chromosomal region as being heterochromatic. Heterochromatic regions when placed adjacent to euchromatic regions by rearrangement breakpoints can suppress the expression of genes located within the euchromatin in a process known as position-effect variegation (2–4). In addition, DNA sequences located within heterochromatin, such as simple satellite sequences, become under-represented in many cells that undergo polyploid development. This phenomenon is particularly dramatic during the development of polytene salivary gland chromosomes in Dipteran insects such as *Drosophila*, where the copy number of a given satellite may be reduced many hundred fold relative to euchromatic regions (reviewed in 5).

While it is generally believed that a distinct chromatin structure, heterochromatin, is primarily responsible for the properties of heterochromatic regions, a detailed molecular description of what heterochromatin is and how its constituent parts cause the cytological and genetic characteristics described is lacking (6). This is due, in part, to the difficulty of manipulating and characterizing large chromosomal regions rich in highly repetitive DNA sequences. These impediments can be overcome by studying small, molecularly tractable model chromosomes such as the 1.3 megabase *Dp(1;f)1187* minichromosome of *Drosophila*. This chromosome contains both euchromatic and heterochromatic domains, and has been used to address questions of heterochromatin structure and function, including the process of heterochromatic sequence under-representation in the polytene chromosomes of larval salivary glands (7–9).

While investigating the copy number of *Dp1187* sequences in the polyploid chromosomes of ovarian nurse and follicle cells of *Drosophila* we discovered that restriction fragments of genomic DNA spanning the euchromatic – heterochromatic junction of *Dp1187* and extending into peri-centromeric sequences had the

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unusual property of being selectively resistant to transfer out of agarose gels during Southern blotting, leading to systematic reductions in *Dp1187*-specific hybridization signals. Evidence is presented in this report consistent with the hypothesis that pericentromeric sequences of *Dp1187* that are incorporated into heterochromatin *in vivo* retain some component of heterochromatic structure during DNA isolation which subsequently causes the reduced transfer efficiency observed *in vitro*.

MATERIAL AND METHODS

Drosophila strains

Information about *Drosophila* genes and nomenclature may be found in Lindsley and Zimm (10). Adult females used for the isolation of ovary DNA in experiments to determine the relative copy number of restriction fragments were from the outcross of males of genotype *X, y/Y; ry⁵⁰⁶/ry⁵⁰⁶; Dp1187, y⁺* to virgin females of genotype *X, y/X, y; ry⁵⁰⁶/ry⁵⁰⁶*. This cross ensured that a single *Dp1187* chromosome was present in virtually all yellow⁺ offspring. Adult females of genotype *X^cX, y/O; Dp1187, y⁺* were obtained from the cross *X^cX, y/O × X^cY, Df(1)259 l(1)J1 y/O; Dp1187, y⁺*. Adult females of genotype *X^cX, y/Y; Dp1187, y⁺* were obtained from the cross *X^cX, y/O × X, y/Y; Dp1187, y⁺*.

Cloned DNAs

Several subclones of X chromosome genomic DNA were used as hybridization probes. The 1.75 kb *HindIII*–*EcoRI* fragment (probe HR1.75) from position –103 of *Dp1187*, the 3.5 kb *EcoRI* fragment (probe R3.5) from position –54 kb of *Dp1187* and the 0.8 kb *BglII*–*PstI* fragment (probe BP0.8) from position

–1.7 kb were described previously (7). A 3.7 kb *XbaI*–*HindIII* fragment (probe XH3.7) that crosses the position of the *sc⁸* junction was subcloned from λ sc¹⁰¹ (11) to create pBSsc¹⁰¹XH3.7. A 1.0 kb *BamHI*–*HindIII* fragment (probe BH1.0) and a 2.6 kb *AseI*–*HindIII* fragment (probe AH2.6) were isolated from pBSsc¹⁰¹XH3.7. The restriction sites delineating all these probes, except HR1.75 and R3.5, are shown on the X chromosome map in Figure 1. The *sc⁸* junction of *Dp1187* was cloned by ligating ovary DNA isolated from females carrying the *Dp1187* chromosome that had been cleaved with *BamHI* and *PacI* into the vector pHD19R (12). Following transformation, colonies bearing the junction fragment were identified by hybridization with probe BH1.0, and the identified clone, pHD-BPDp, verified by restriction mapping. The corresponding clone from the unrearranged X chromosome was similarly recovered and named pHD-BPX. DNA sequencing was carried out starting at the *BamHI* site of both clones. The *sc⁸* junction was identified as the point at which the sequences of the two clones diverged. The sequences can be retrieved from GenBank under accession numbers L32746 for *Dp(1;f)1187* and L32747 for the X chromosome.

Preparation and digestion of DNA in agarose inserts

Agarose-embedded DNA from adult ovaries was prepared as described previously (9). Ovaries were dissected from a large number of animals at one time and pooled for preparation in agarose. For individual digests DNA from 5–10 pairs of ovaries was used.

Electrophoresis and DNA hybridization

Agarose inserts containing restriction-digested DNA were placed into the wells of 0.8% Seakem GTG (FMC) agarose gels in 0.5× TBE buffer (13). DNA was fractionated by CHEF electrophoresis (14) at an angle of 120°, 14°C, 6 V/cm and using a variety of pulsing regimes depending on the experiment (see figure legends). Conventional electrophoresis was also used as appropriate for smaller size fragments. For both CHEF and conventional electrophoresis gels were typically 0.7–0.9 cm in thickness, although, as discussed in the Results, thinner gels were also used. After electrophoresis, the DNA was stained with 0.5 µg/ml ethidium bromide in 0.5× TBE buffer and photographed on a UV light box. Transfer inhibition was unaffected by ethidium bromide staining or photography. After staining, the DNA was transferred to Gene Screen filters (New England Nuclear) by ascending capillary Southern blotting. Our standard Southern blotting protocol was as follows. Gels were incubated 1×15 min in 0.25 N HCl, rinsed briefly in water, incubated 1×30 min in 0.4 N NaOH, 0.8 M NaCl, rinsed briefly in water, incubated for 2×10 min in 0.025 M NaHPO₄, and blotted in 5× SSPE (13) using a 5 cm thickness of GB004 blotting paper (Schleicher and Schuell) and 1–2 g/cm² of weight applied to the top of the blotting paper. After blotting, the membrane was incubated 1×5 min in 5× SSPE and UV crosslinked using 120 mJ/cm². Many variations of this Southern blotting protocol were tried to assess their impact on *Dp1187* transfer inhibition (see also Results). They included extended depurination times, transfer in alkaline buffer, gel neutralization in 0.5 M Tris, pH 7.5, 1.5 M NaCl before blotting in 20× SSPE, adding an additional post-blot denaturation of the filter, and use of charged Gene Screen Plus membranes (New England Nuclear). Hybridization was done using the protocol of Church and Gilbert (15). Probes were made

Figure 1. Structure of *Dp1187* and restriction maps of *sc⁸* regions of *Dp1187* and the X chromosome. A map of the *Dp1187* minichromosome is shown at top. The coordinate system used (in kilobases) is indicated where 0 kb is defined as the position of the *sc⁸* rearrangement junction. Euchromatic sequences are shown as a line and heterochromatic sequences as a stippled bar. An 'island' of complex sequences located between approximately +50 kb and +90 kb is shown in black (8). The open oval represents the centromere, although its exact location is unknown. Relevant restriction sites are shown within a portion of the *Dp1187* chromosome near the *sc⁸* junction and in the corresponding euchromatic region of a normal X chromosome. Note the difference in scale between the *Dp1187* and X chromosome maps. The restriction fragments of *Dp1187* analyzed for relative copy number in Figure 2 are shown as black lines below the *Dp1187* map. A = *AseI*; B = *BamHI*; G = *BglII*; H = *HindIII*; N = *NotI*; P = *PacI*; R = *EcoRI*; S = *SfiI*; T = *PstI*; X = *XbaI*.

from DNA fragments isolated in low melting temperature agarose and labeled with ^{32}P by random priming (16). Filters were exposed to Kodak XAR-5 film at -80°C with intensifying screens and quantitated directly using a Molecular Dynamics Phosphorimager and Imagen software.

The in-gel hybridization procedure was modified from the procedure of Purrello and Balazs (17). After the DNA in the agarose gel was stained with ethidium bromide and photographed, the gel was placed on GB002 filter paper (Schleicher and Schuell) and placed on a flat metal surface in a 65°C gravity convection oven and allowed to dry completely. Drying required $\sim 2-3$ h. Dried gels could be stored at 4°C for at least a few days, probably much longer. The gel was rehydrated in water for 2×5 min, denatured in 0.4 N NaOH, 0.8 M NaCl for 30 min, neutralized in 0.5 M Tris, pH 7.4, 0.8 M NaCl for 30 min and rinsed in water for 5 min. Prehybridization, hybridization and washes were done using the same Church and Gilbert protocol (15) used for filter hybridizations except the prehybridization and washes were for longer times. Prehybridization was for 3–4 h. Hybridization was for ~ 15 h with $\sim 2 \times 10^6$ c.p.m./ml of probe labeled with ^{32}P by random priming to specific activities of $\sim 1 \times 10^9$ c.p.m./ μg . Low stringency washes were 1×20 min and 1×1 h. High stringency washes were 2×2 h. After washing, excess wash buffer was blotted off the gel which was then wrapped in Saran Wrap and exposed to Kodak XAR-5 film at -80°C with intensifying screens or to stored-phosphor imaging plates (Molecular Dynamics).

RESULTS

Quantitative Southern blot and in-gel hybridization analysis

The origin of *Dp(1;f)1187* from a normal X chromosome has been described (7), and its overall structure is shown in Figure 1. Sites along the chromosome are designated according to a coordinate system in which position 0 kb corresponds to the location of the *sc*⁸ inversion junction that was acquired during minichromosome construction. Peri-centromeric sequences derived from deep within the basal region of the X chromosome lie to the right of the junction and are designated with positive kb values. Euchromatic sequences from the tip of the X lie to the left of the junction and are designated with negative kb values. Restriction maps for the *sc*⁸ region of *Dp1187* and the homologous region of euchromatic sequences located on the X chromosome are illustrated.

Dp1187 sequences have been demonstrated previously to be under-represented in polytene salivary gland chromosomes (7). We wanted to determine whether *Dp1187* sequences were also under-represented in the polyploid chromosomes of ovarian nurse cells and follicle cells. DNA from these cells constitutes the majority of DNA in whole adult ovaries (18). Under-representation of peri-centromeric sequences, in particular, might be expected, since satellite sequences of the normal chromosomes are under-represented in these cells (19). DNA was isolated from ovaries, cut with restriction enzymes, fractionated by agarose gel electrophoresis, and analyzed by both Southern blot hybridization as well as by hybridization done directly within agarose gels (in-gel hybridization). The reason for doing in-gel hybridization analysis will be apparent after considering the results from the analysis by Southern blotting. Unique sequence probes from the *sc*⁸ junction region hybridized to both *Dp1187*-specific and X-specific restriction fragments for each

digest. The ratio of hybridization signals from these fragments provided a measure of the representation of sequences near and within peri-centromeric sequences of *Dp1187* relative to entirely euchromatic sequences from the normal X chromosome. Approximately 1100 kb of *Dp1187* spanning the *sc*⁸ junction was analyzed using six different digests (Figure 1). All DNA samples were prepared and digested in agarose inserts, allowing analysis of the larger restriction fragments extending furthest into

Figure 2. Quantitative Southern blot and in-gel hybridization analysis of *Dp1187*.

(A) Agarose-embedded ovary DNA from adult females of genotype *X/X; Dp1187* was prepared as described (see Materials and Methods). Southern blot hybridizations (blot) and in-gel hybridizations (gel) are illustrated for six different digests. The numbers above the panels indicate the centromere-proximal endpoint of the restriction fragment relative to the *sc*⁸ breakpoint. The *Dp1187*-specific (Dp) and X-specific (X) bands are indicated and their sizes in kilobase pairs (kb). Samples for -103 were digested with *Hind*III, fractionated by conventional electrophoresis, and probed with HR1.75. Samples for -54 were digested with *Eco*RI, fractionated by conventional electrophoresis, and probed with R3.5. Samples for $+0.128$ were digested with *Ase*I. Samples for $+70$ were digested with *Hind*III. Samples for $+1000$ were digested with *Sfi*I or *Not*I. The *Sfi*I digest generated a 115 kb X-specific band, and *Not*I a 240 kb X-specific band. The $+0.128$, $+70$ and $+1000$ samples were fractionated by CHEF electrophoresis and probed with AH2.6. The pulse regime used was 50–130 s linearly ramped for a total of 8 h. The larger molecular weight bands in -103 are not a result of partial digestion by *Hind*III, but are due to cross-hybridization of the probe with heterogeneous high molecular weight molecules in this digest. (B) Southern blot and in-gel hybridizations as illustrated in Figure 2A were quantitated using stored-phosphor imaging plates. The ratio of *Dp1187*-specific hybridization signal to X-specific hybridization signal was calculated for each digest, and results from independent experiments are indicated with '+' for Southern hybridizations and 'o' for in-gel hybridizations. Ratios are plotted on a log scale versus the position of the centromere-proximal endpoint of the restriction fragment assayed. The lines connect the calculated mean of the independent determinations. The boxed numbers above the in-gel samples and below the blot samples indicate the number of independent measurements made for that restriction fragment. The expected *Dp1187*:X ratio of 0.5 for the genotype *X/X; Dp1187* is shown as a horizontal dashed line. Southern blot data from experiments in which transfer was done under alkaline conditions are indicated by the arrows to the right of the '+'. All other experiments used transfer in neutral buffers.

peri-centromeric sequences by pulsed-field electrophoresis. Hybridization signals were quantitated using stored-phosphor imaging plates, and the ratio of *Dp1187*-specific hybridization signal to *X*-specific hybridization signal for each digest was calculated. The results of one series of Southern blot and in-gel hybridization analyses are illustrated in Figure 2A. The ratios measured from many independent analyses are illustrated graphically in Figure 2B. Since the female animals used for this analysis were of genotype *X/X; Dp1187*, the expected *Dp1187:X* ratio of hybridization signals was 0.5.

Figure 3. *Dp1187* restriction fragments were selectively retained in agarose gels during Southern blot transfer. (A) Agarose-embedded ovary DNA from adult females of genotype *X/X; Dp1187* was prepared as described (see Materials and Methods). The DNA was restricted with *HindIII* and fractionated by CHEF electrophoresis with a pulse time of 10 s for a total of 12 h. After ethidium bromide staining and photography, the gel was subject to Southern blot hybridization (blot), in-gel hybridization (in-gel), or in-gel hybridization after Southern transfer (post-blot). Unique sequence probe AH2.6 hybridized to *Dp1187*-specific (*Dp*) and *X* chromosome-specific fragments (*X*) of sizes in kb shown to the right. The Southern blot, in-gel, and post-blot hybridization experiments were done in parallel and autoradiographic exposures were done for approximately the same length of time. (B) Agarose-embedded DNA from females of genotype *X/X; Dp1187* was prepared from adult ovaries, restricted with *EcoRI*, and fractionated by CHEF electrophoresis as described for (A). One lane of the gel was analyzed by Southern blot hybridization using probe XH3.7 (panel 1) which hybridizes to *Dp1187*-specific (*Dp*) and *X* chromosome-specific fragments (*X*) of sizes in kb shown to the right. The hybridization signal to the *Dp1187*-specific band was very reduced and was not detectable on the exposure illustrated. Another lane of the same agarose gel containing *EcoRI*-digested DNA was excised and incubated with *BglII*, restricting the DNA within the gel with a second enzyme. The gel slice was then molded into a second agarose gel, and the now double-digested DNA fractionated by conventional electrophoresis in a direction perpendicular to the first electrophoresis (arrows indicate direction of electrophoresis). The second dimension gel was subject to Southern blot hybridization using probe BP0.8 (panel 2) which hybridizes to *Dp1187*-specific and *X*-specific fragments of sizes in kb shown at the top of the panel. Both *X*-specific and *Dp1187*-specific bands of expected hybridization ratio were observed. w = position of well.

The ratios measured by Southern blotting for restriction fragments located within euchromatin at positions -103 and -54 varied no more than 2-fold from the expected ratio of 0.5. In contrast, ratios measured by Southern blotting for restriction fragments that crossed the *sc*⁸ junction extending to positions $+0.128$, $+70$ and $+1000$ were markedly lower than 0.5, as well as being more variable (Figure 2A and Figure 2B, values marked '+'). The *Dp1187:X* ratios measured for the *HindIII* fragment extending to $+70$ and for the *NotI* and *SfiI* fragments extending to position $+1000$ were on average 10- and 50-fold lower than predicted, respectively (Figure 2B). Examination of the autoradiograms revealed that the reduced ratios were a consequence of a progressive loss of *Dp1187*-specific hybridization signal (Figure 2A, compare *Dp1187*-specific signal for 'blot' data at positions $+0.128$, $+70$ and $+1000$). The absolute loss of *Dp1187*-specific hybridization signal for some measurements was so severe as to preclude detection of any signal except on very prolonged exposures (Figure 2A, $+1000$ lanes in 'blot' panel, and Figure 3A, panel marked 'blot'). This extreme reduction in *Dp1187* hybridization signal was also observed for the *EcoRI* restriction fragment which extends to $+78$ (Figure 2B and Figure 3B, panel 1) and for four other restriction fragments that were assayed that extend to positions $+55$ to $+90$ (data not shown). Signal missing from full-length restriction fragments was not accounted for by hybridization to any new DNA species of altered mobility located elsewhere on the gels (Figure 2A).

Hybridization to DNA directly in agarose gels after electrophoretic fractionation (in-gel hybridization) was tried in an attempt to identify *Dp1187* signal missing on the Southern blots. The in-gel hybridizations revealed increased levels of *Dp1187*-specific hybridization signal at positions in the gel of predicted full-length fragments (Figure 2A, compare lanes for $+70$ and $+1000$ in panels marked 'blot' versus 'gel'). This observation was most dramatic with the *HindIII* fragment extending to $+70$. In Figure 3A is illustrated a Southern blot hybridization that gave a *Dp1187:X* ratio of 0.013 (panel 'blot') and an in-gel hybridization that gave a ratio of 0.37 (panel marked 'in-gel'). The increased ratio was clearly a consequence of increased hybridization to the *Dp1187*-specific fragment. On average the *Dp1187:X* hybridization ratios measured by in-gel hybridization for fragments extending to $+0.128$ and $+70$ were at or close to the predicted value of 0.5 (Figure 2B, values marked 'o'). The ratios measured by in-gel hybridization for the *NotI* and *SfiI* fragments extending to $+1000$ were reproducibly greater than ratios measured by Southern blotting, but were still significantly below the predicted value of 0.5 (Figure 2A and B).

Prominent labeling of the wells was commonly observed for the in-gel hybridizations (Figure 2A, lanes marked 'gel'). The labeling, however, was highly variable and observed in all samples, including the euchromatic fragments at -54 and -103 , suggesting that the signal is a non-specific artifact of the in-gel protocol. This non-specific labeling could have obscured real hybridization to *Dp1187* or *X* DNA trapped in the wells, however, a two-dimensional Southern blot analysis capable of identifying such DNA, discussed below, revealed no significant labeling of the wells (Figure 3B).

Measurements of *Dp1187:X* hybridization ratios by Southern blotting and in-gel hybridization showed little or no difference for euchromatic fragments located at positions -103 and -54 (Figure 2A and 2B). In contrast, the two techniques gave significantly different hybridization ratios for restriction fragments

that extended across the sc^8 junction and into peri-centromeric sequences (Figure 2B, compare '+' to 'o'). The difference in response of euchromatic versus heterochromatic fragments demonstrates that fragments spanning the sc^8 junction have physical properties distinct from fragments located entirely within euchromatin.

Hybridization to agarose gels after their use for Southern blotting revealed that hybridization signal was lost from Southern blots because fragments that extended across the sc^8 junction

Figure 4. Inhibited transfer of *Dp1187* restriction fragments was not caused by an inherent property of satellite sequences. (A) Restriction maps of the *Bam*HI–*Pac*I fragments from the *Dp1187* and *X* chromosomes analyzed as cloned DNA are shown on top, and the *Ase*I fragments from the *Dp1187* and *X* chromosomes analyzed in genomic DNA are shown below. The position 0 kb is the position of the sc^8 rearrangement junction. Euchromatic sequences are shown as a line and heterochromatic sequences as a stippled bar. The reiterated *Ase*I sites in the cloned *Bam*HI–*Pac*I fragment from the *Dp1187* chromosome arise from repeats of the 1.688 satellite sequences. A = *Ase*I; B = *Bam*HI; P = *Pac*I. (B) *Dp*:*X* hybridization ratios for the cloned *Bam*HI–*Pac*I fragments (shaded bars) were determined as follows. Plasmids pHD-BPDp and pHD-BPX, containing *Bam*HI–*Pac*I fragments that cross the position of the sc^8 junction from the *Dp1187* and *X* chromosomes, respectively, were isolated from *E. coli* by the alkaline lysis method and restricted with *Bam*HI and *Pac*I. Samples from the *Dp1187* and *X* digests were combined, and serial dilutions of the DNA were fractionated by conventional electrophoresis. DNA was analyzed by both Southern blot hybridization (blot) and in-gel hybridization (gel) using probe BH1.0. The *Dp1187*-specific and *X*-specific hybridization signals were quantitated using stored-phosphor imaging plates and *Dp1187*:*X* hybridization ratios were calculated. The expected values for the hybridization ratios for the cloned DNA fragments were calculated from the empirically measured mass amount of the DNA fragments in gels as measured from high resolution negatives of ethidium bromide-stained gel photographs. These measurements were standardized to a value of 0.5 (shown with a dashed line) to facilitate comparison between the cloned and genomic DNA samples. *Dp1187*:*X* hybridization ratios for the *Ase*I fragments in genomic DNA (striped bars) were determined as described for Figure 2 by both Southern blot (blot) and in-gel hybridization (gel) using probe AH2.6. The predicted *Dp1187*:*X* hybridization ratio for genomic *Ase*I fragments was 0.5, the ratio expected for the genotype *X*:*X*; *Dp1187*. The height of each bar indicates the mean calculated from independent measurements. The number of independent measurements is indicated above each bar. Standard deviations are shown.

were selectively retained in the agarose gel during the blotting process, failing to transfer to the membrane support (Figure 3A, note panel marked 'post-blot'). Even when blotting conditions were used that left no detectable residual *X*-specific hybridization signal in the post-blot gel, some amount of *Dp1187*-specific hybridization was always observed remaining in the gel (data not shown).

The unusual property of *Dp1187* restriction fragments to be retained in agarose gels during Southern blotting was independently demonstrated using a two-dimensional agarose gel electrophoresis approach (Figure 3B). The *Eco*RI restriction fragment of *Dp1187* which crosses the sc^8 junction and extends 78 kb into peri-centromeric sequences gave a severely reduced hybridization signal as assayed by Southern blotting (Figure 3B, lane 1). Its presence in the gel prior to Southern transfer, however, was revealed by first fractionating *Eco*RI-digested DNA by electrophoresis and then digesting an entire lane of the agarose gel with a second enzyme, *Bgl*II. *Bgl*II cuts internal to *Eco*RI entirely within euchromatic sequences and is also polymorphic for the *Dp1187* and *X* chromosomes (see Figure 1). When the

Figure 5. The *Y* chromosome suppressed transfer inhibition. (A) The percentage of *y*⁻ triple-row bristles on the wings of 10 females of genotype *XX*, *y*/*O*; *Dp1187*, *y*⁺ and of 10 females of genotype *XX*, *y*/*Y*; *Dp1187*, *y*⁺ were measured. Each + in the graph is the value from one female, and the line indicates the mean value which is shown to the right. (B) Agarose-embedded DNA was prepared from the ovaries of the same females used to measure yellow variegation shown in (A). The DNA was restricted with *Ase*I, fractionated by conventional electrophoresis and analyzed either by in-gel hybridization (black bars) or by Southern blot hybridization (gray bars). Hybridization results were quantitated using stored-phosphor imaging plates and the *Dp1187*:*X* hybridization ratios calculated.

double-digested DNA was then separated by a second electrophoresis perpendicular to the first, a *Dp1187*-specific *Bgl*III band of the expected 1.5 kb size was observed migrating at a position consistent with the presence of an 85 kb *Eco*RI fragment in the first dimension gel. In contrast to the 85 kb *Eco*RI fragment, hybridization signal to the 1.5 kb *Dp1187 Bgl*III fragment was not reduced, and the *Dp1187:X* hybridization ratio was 0.55 (Figure 3B).

The two-dimensional agarose gel electrophoresis experiment demonstrated two things. First, it confirmed the in-gel hybridization results (Figures 2 and 3A) demonstrating that *Dp1187* DNA fragments were present in gels after electrophoresis at predicted abundance relative to the *X* chromosome but did not transfer during Southern blotting. Second, it illustrated that while the full-length 85 kb *Eco*RI fragment was resistant to transfer, the euchromatic part of that *Eco*RI fragment which included the sequences of the *Bgl*III segment could still be cut, further fractionated by electrophoresis, transferred without inhibition, and detected by hybridization. We interpret this result to mean that the mechanism leading to transfer inhibition is localized to, or arises from, that portion of the *Eco*RI fragment containing peri-centromeric sequences. The property associated with these sequences can act in *cis* to inhibit transfer of the entire fragment during blotting.

The severe loss of *Dp1187* hybridization signal observed for the *Hind*III fragment and other fragments extending furthest into peri-centromeric sequences was not a trivial consequence of the larger size of these fragments causing reduced transfer efficiency. Restriction fragments of larger size from euchromatic regions of the *X* chromosome, *Dp1187*, and the autosomes were routinely detected without apparent loss of hybridization signal (Figure 2A, *X*-specific bands; 20 and data not shown).

Many parameters of the Southern blotting protocol were varied to determine to what extent they would influence transfer of *Dp1187* restriction fragments. Transfer at a variety of salt concentrations, in alkaline buffers (see Figure 2, measurements highlighted by arrows), by electroblotting, transfer to a variety of charged and neutral membranes, and acid depurination for up to 120 min all failed to consistently reduce the selective retention of *Dp1187* fragments (data not shown; see also Materials and Methods). To see whether insufficient deprotection of the samples was contributing to transfer inhibition, DNA samples were treated with 6 mg/ml pronase for 16 h in addition to the normal 1 mg/ml proteinase K treatment, but all samples gave the same reduced hybridization regardless of the protease treatment used (data not shown).

While none of the experimental conditions tried mitigated the transfer inhibition entirely, some parameters did have effects and likely contributed to the variability in measured ratios reported in Figure 2B. Both gel thickness and the weight applied during ascending capillary blotting had some effect, with thinner gels and less weight both tending to result in improved transfer (data not shown). It also appeared that *Dp1187*-specific fragments transferred better when they were located in a region of gel in which the total DNA concentration was increased as a consequence of electrophoretic compression. For example, the pulsed-field electrophoresis conditions used for the gel illustrated in Figure 2A (panel marked 'blot') lead to substantial compression and a *Dp1187:X* hybridization ratio of 0.11 was observed. In contrast, the pulsed-field electrophoresis conditions used for the gel illustrated in Figure 3A (panel marked 'blot') were optimized for maximal separation of DNA in the region of the 80 kb

fragment and a *Dp1187:X* hybridization ratio of 0.013 was measured.

Transfer inhibition was not caused by an inherent physical property of nucleotide sequence

We tested the premise that repetitive satellite sequences had an inherent ability *in vitro* to inhibit the transfer of restriction fragments on which they were located. A number of observations argued against this hypothesis. Transfer under alkaline conditions, which would inhibit renaturation artifacts, had no effect (Figure 2B, '+' values highlighted by arrows), and the *Ase*I fragment, which demonstrated consistently reduced transfer efficiency (Figure 2B, position +0.128), contains no internally repetitive sequences (see below and Figure 4A). Nonetheless, the transfer efficiency of a cloned restriction fragment from *Dp1187* that contained extensive repetitive satellite sequences was measured.

The *Bam*HI–*Pac*I fragment spanning the *sc*⁸ junction was cloned from *Dp1187* as well as the normal *X* chromosome (see Material and Methods). This fragment from *Dp1187* includes 1 kb of euchromatic sequences and 2.5 kb of peri-centromeric sequences (Figure 4A). Sequencing revealed that euchromatic sequences had been joined at the *sc*⁸ junction to at least six tandem repeats of an abundant satellite DNA known as the 1.688 or 359 bp satellite. This satellite comprises ~4% of *Drosophila* genomic DNA (21) and had previously been localized exclusively to the basal region of the *X* chromosome by *in situ* hybridization (22). The unit length of the repeats on the *Bam*HI–*Pac*I clone is slightly larger than the canonical 359 bp owing to an internal repeat of 65 bp (data not shown). On the *Dp1187* minichromosome the 1.688 repeats are unlikely to extend beyond position +50 kb, since many restriction sites that are absent from *Drosophila* satellite DNAs are found in this region (7; Figure 1).

Transfer efficiency of the cloned *Bam*HI–*Pac*I fragments was determined by comparing empirical measurements of *Dp1187:X* hybridization ratios to the hybridization ratios predicted based on physical measurements of the cloned fragments by ethidium bromide staining (see Figure 4 legend). The results for the cloned fragments were then compared with ratios measured for the *Ase*I fragments from genomic DNA (Figure 4). Unfortunately, a direct comparison of cloned *Bam*HI–*Pac*I fragments to *Bam*HI–*Pac*I fragments in genomic DNA was precluded by the inability to obtain complete digestion with these enzymes in agarose inserts. In contrast to the reduced hybridization ratios measured in genomic DNA for the *Ase*I fragments, ratios measured for the cloned *Bam*HI–*Pac*I fragments were at or exceeded (by ~30%) predicted values, showing no evidence for reduced transfer of the *Dp1187* fragment (Figure 4, compare shaded bars to striped bars). In addition, ratios measured by in-gel hybridization for the cloned fragments were close to or slightly lower, on average, than ratios measured by Southern blotting. This contrasts with ratios for the genomic *Ase*I fragments measured by in-gel hybridization, which were consistently and significantly higher than ratios measured by Southern blotting (Figure 4B, compare 'blot' and 'gel'). These results demonstrate that sequences of the 1.688 satellite repeats did not have an inherent ability to inhibit the transfer of the cloned *Dp1187*-specific *Bam*HI–*Pac*I fragment. These results are also consistent with the general conclusion that simply the presence of repetitive satellite sequences on *Dp1187* restriction fragments did not cause transfer inhibition, and that something else, something associated with genomic DNA from flies but not cloned DNA from *E. coli*, was responsible for inhibition.

Influence of the *Y* chromosome

If transfer inhibition derives from an epigenetic structure or complex found only in genomic DNA, as the results presented in the preceding section suggest, and given the association of pericentromeric satellite sequences with heterochromatic regions of chromosomes (1), then perhaps the incorporation of *Dp1187* pericentromeric sequences into heterochromatin *in vivo* in some way leads to the transfer inhibition observed *in vitro*. If this hypothesis is correct, then genotypes that are known to suppress heterochromatin formation and/or function, such as genetic suppressors of position-effect variegation (2–4), might be expected to reduce the degree to which *Dp1187* fragments are retained in agarose gels during blotting and thereby increase *Dp1187:X* hybridization ratios.

The ability of the heterochromatic *Y* chromosome to strongly suppress, *in trans*, position-effect variegation of euchromatic genes located near euchromatic/heterochromatic junctions is well characterized (23). For this reason, females of genotypes *X⁺X/O*; *Dp1187* and *X⁺X/Y*; *Dp1187* were constructed. DNA from their ovaries was isolated, digested with restriction enzymes, fractionated by electrophoresis, and analyzed by Southern blot and in-gel hybridization to determine whether addition of the *Y* chromosome to the genotype would increase *Dp1187:X* hybridization ratios.

The influence of the *Y* chromosome in these genotypes was monitored phenotypically by measuring the extent of variegated expression of the yellow gene located at –20 kb on the *Dp1187* chromosome. Expression of the yellow gene on *Dp1187* was required for the pigmentation of triple-row bristles on the wing. Addition of the *Y* chromosome to the genotype did suppress variegated expression of the yellow gene in the triple-row bristle cells significantly (Figure 5A), consistent with the known influence of the *Y* chromosome on position-effect variegation.

Dp1187:X hybridization ratios measured by both Southern blot and in-gel hybridizations for a variety of digests showed a small but consistent increase in females with an added *Y* chromosome in their genotype. Results for the *AseI* fragment (+0.128) are shown in Figure 5B. Although modest, seeing any effect suggests that some aspect of *Dp1187* structure that is sensitive to the *Y* chromosome *in vivo* can influence the level of *Dp1187*-specific hybridization measured *in vitro*. Given the known influence of the *Y* chromosome on heterochromatin, this data supports the model that it is the incorporation of pericentromeric sequences of *Dp1187* into heterochromatin *in vivo* that can subsequently influence the efficiency of Southern blot transfer *in vitro*.

DISCUSSION

We demonstrate in this report that genomic DNA fragments spanning the euchromatic–heterochromatic junction of the *Drosophila* minichromosome *Dp(1;f)1187* were selectively retained in agarose gels during DNA Southern blotting protocols. This property was persistently associated with the *Dp1187* DNA, despite attempts to ameliorate the effect by altering DNA preparation and Southern blotting protocols. Transfer inhibition was associated with the pericentromeric sequences contained on these genomic fragments, yet was not a property of at least some of those same sequences on cloned DNA. Finally, the degree of transfer inhibition was partially suppressed by addition of a *Y* chromosome to the genotype. Based on these observations and the fact that pericentromeric regions of most eukaryotic

chromosomes are associated with cytologically and genetically defined heterochromatin, we proposed that as a consequence of their incorporation into heterochromatin the pericentromeric sequences of *Dp1187* are subject to some alteration *in vivo* that persists during DNA isolation and subsequently interferes with DNA transfer *in vitro*.

The genomic DNA used for the analysis reported here was isolated from polyploid nurse and follicle cells of the ovary. The property of *Dp1187* DNA to be retained in agarose gels during Southern blotting, however, is not a property unique to polyploid chromosomes. We have also observed transfer inhibition of *Dp1187* DNA isolated from predominantly diploid tissues such as imaginal discs and early embryos (unpublished data). The observation of transfer inhibition in DNA from diploid cells suggests that the property of DNA leading to transfer inhibition is not a peculiarity of polyploid chromosomes and may reflect a more general aspect of heterochromatin structure.

What alteration might the pericentromeric sequences of *Dp1187* be subject to that would become or remain associated with the DNA during isolation, subsequently interfering with Southern blotting? The alteration must be protease-resistant and is likely phenol-resistant as well, since the transfer of *Dp1187* DNA prepared in solution using phenol extraction was also inhibited (data not shown). Proteins have been identified that are tightly and preferentially bound to satellite and repetitive DNA sequences and that are also protease- and phenol-resistant (24–26). Perhaps these proteins, or proteins with these characteristics, are a normal constituent of heterochromatin, tightly binding pericentromeric sequences of *Dp1187* *in vivo*, remaining bound during isolation and subsequently entangling the DNA within the agarose gel during Southern transfer. Topoisomerase II could also play a role in this phenomenon as it interacts covalently with DNA and has been shown to bind 1.688 satellite sequences as are found on the *Dp1187* chromosome (27). We are presently attempting to identify proteins or peptides that co-purify with genomic DNA in our preparations. Such proteins would be candidates for involvement in transfer inhibition.

Non-protein modifications of *Dp1187* DNA must also be considered as possible sources of the inhibitory effect. While earlier attempts to identify modified nucleotides in *Drosophila* were unsuccessful (28), satellite sequences may have escaped analysis due to their potential for selective loss during extraction with organic solvents (29, 30), or perhaps the modification is of such unexpected structure as to be missed by conventional analysis. Particularly germane to this report is the recent discovery of a novel DNA modification, β -D-glucosyl-hydroxymethyluracil, in *Trypanosoma brucei*. This modification localizes to repetitive telomere DNA and may be involved in repression of subtelomeric expression sites for VSG genes (31). This particular modification is not found in *Drosophila* DNA (31), but perhaps repetitive sequences in heterochromatin of *Drosophila* contain some other novel modification that causes transfer inhibition *in vitro*. It is not apparent how any known DNA modification would mediate the transfer inhibition that we have identified, so if DNA modification is involved, it may be of a previously uncharacterized nature.

The initial objective of this study was to identify quantitative reductions of pericentromeric sequences of *Dp1187* in ovary DNA. Identification of such reductions would provide an opportunity to investigate the process that causes underrepresentation of satellite sequences during formation of polyploid

chromosomes (reviewed in 5). While reductions in *Dp1187*-specific hybridization signals were identified using standard Southern blotting protocols, unexpectedly, a large part of those reductions were a consequence of the selective retention of *Dp1187* restriction fragments in agarose gels during the blotting process, and were not due to actual *in vivo* loss of *Dp1187* sequences (Figures 2 and 3). Thus, reductions in hybridization signal do not always reflect true *in vivo* loss of DNA. This observation has obvious implications for quantitative interpretation of Southern blot hybridization data, especially when the DNA being examined is within or near heterochromatin. We are examining other regions of the *Drosophila* genome to determine the generality of this phenomenon with respect to heterochromatic regions other than the *sc*⁸ junction of *Dp1187*.

While transfer inhibition accounted for much of the observed reduction in *Dp1187*-specific hybridization signal, actual *in vivo* sequence under-representation may still occur on the *Dp1187* chromosome in ovarian nurse and follicle cells. While *Dp1187:X* hybridization ratios measured by in-gel hybridization were consistently higher than ratios measured by Southern blotting, they were often still below predicted values. For example, ratios measured by in-gel hybridization for the *AseI* fragment (Figure 2B, position +0.128, and Figure 4B, striped bar marked 'gel') and the *NotI* and *SfiI* fragments (Figure 2, position +1000) were consistently lower than expected. It is also likely that under-representation occurs for the *Dp1187* chromosome in polytene nuclei of larval salivary gland cells, since reductions in *Dp1187* hybridization signal previously measured by Southern blotting (7) were also observed by in-gel hybridization (unpublished data).

Analysis of euchromatic sequences in genomic DNA by Southern blot hybridization typically gives consistent, straightforward results regardless of the location of the sequences within the genome. By contrast, analysis of peri-centromeric sequences of *Dp1187* has revealed that some regions of the *Drosophila* genome have very unexpected properties that complicate orthodox molecular analysis. To the extent that the analysis of *Dp1187* is revealing properties general to eukaryotic chromosomes, the results presented here have implications for analysis of genome structure with respect to heterochromatic regions such as centromeres and telomeres, and, more interestingly, suggest that the poorly understood heterochromatic domains of eukaryotic chromosomes likely conceal interesting and as yet uncharacterized biological phenomenon.

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