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Genetics

Ribosomal DNA and Stellate gene copy number variation on the Y chromosome of *Drosophila melanogaster*

(molecular evolution/polymorphism/multigene family/copy number estimation)

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ABSTRACT **Multigene families on the Y chromosome face an unusual array of evolutionary forces. Both ribosomal DNA and Stellate, the two families examined here, have multiple copies of similar sequences on the X and Y chromosomes. Although the rate of sequence divergence on the Y chromosome depends on rates of mutation, gene conversion and exchange with the X chromosome, as well as purifying selection, the regulation of gene copy number may also depend on other pleiotropic functions, such as maintenance of chromosome pairing. Gene copy numbers were estimated for a series of 34 Y chromosome replacement lines using densitometric measurements of slot blots of genomic DNA from adult *Drosophila melanogaster*. Scans of autoradiographs of the same blots probed with the cloned alcohol dehydrogenase gene, a single copy gene, served as internal standards. Copy numbers span a 6-fold range for ribosomal DNA and a 3-fold range for Stellate DNA. Despite this magnitude of variation, there was no association between copy number and segregation variation of the sex chromosomes.**

A diverse array of forces is at play that can influence the number of copies of repeated genes on the Y chromosome. In *Drosophila*, the processes of amplification, unequal crossing-over, and unequal sister chromatid exchange appear to be regulated by complex mechanisms of compensation and magnification. To begin to understand the evolutionary aspects of copy-number regulation, the structure and regulation of the gene families must be understood. The 18S and 28S rRNA genes (rDNA) of *Drosophila melanogaster*, encoded on a single transcription unit, are distributed into two cytologically identifiable clusters known as nucleolus organizers (NOs) (1, 2). They are located on the X chromosome near the centromere and on the short arm of the Y chromosome, with estimated numbers of copies at each location ranging from 100 to 240 in laboratory stocks (3). Low copy number is associated with the bobbed (bb) phenotype, characterized by delayed development, abdominal etching, and thin short bristles. If the copy number falls below about 15% of the wild-type number, embryonic lethality results (3). The rDNA unit has been cloned (4, 5), and its molecular structure has been extensively analyzed. The sequence of the complete rDNA repeat reveals a structure with an intergenic sequence of 3632 ± base pairs (bp), an external transcribed spacer (864 bp), the 18S unit (1995 bp), and a 28S unit (3945 bp) (6). Between the 18S and 28S genes is an internal transcribed spacer that encodes a 5.8S rRNA and a 2S rRNA. The partially transcribed intergenic sequence contains a series of 240-bp *Alu* I repeats, each of which may serve as an enhancer of transcription (7). This view is supported by the observation that lines of *Drosophila* with rapid developmental rates tend to have longer intergenic sequence regions (8). Further evidence for the functional contraints of the intergenic sequence comes from the high level of sequence conservation among species of *Drosophila* (9). Within the 28S unit there can be type I or type II insertion sequences. Type I sequences interrupt the 28S unit in about 60% of the X chromosome copies, and they vary in length from 0.5 to 6.5 kilobases, whereas type II inserts occur in about 15% of the 28S rDNA units on both the X and Y chromosomes (10, 11). The two insertion sequences are highly site specific, with points of integration that are 51 bp apart (12). Transcripts of the interrupted genes can be detected, but they occur at very low levels and fail to produce mature rRNA, even in bobbed mutants (13, 14). The severity of the bb phenotype is inversely correlated with the copy number of rRNA genes lacking inserts (15).

The influence of natural selection on copy number is modulated by compensation and magnification. Compensation refers to differential replication of rDNA such that the rDNA content of XX and X0 flies is the same, indicating a 2-fold higher level of amplification in the X0 flies (16). Compensation is a purely somatic phenomenon, whereas magnification results in increased germ-line copy numbers. Magnification is most frequently observed among the gametes of males that are low in rDNA on both sex chromosomes. X–Y chromosomal translocations reveal that part of the long arm of the Y chromosome, distinct from NO, is necessary for magnification in males and that females that have this part of the Y^L chromosome also magnify (17, 18). Magnification results in amelioration of the bobbed phenotype, so active genes are involved, but whether genes lacking the insertion sequences are preferentially amplified remains controversial (11, 19). The dramatic changes in copy number associated with magnification appear to occur only when there is a physiological demand for rRNA.

Another repeated gene family that has members on both sex chromosomes is Stellate (*Ste*). In X0 males, which fail to undergo normal spermatogenesis, primary spermatocytes contain either needle- or star-shaped proteinaceous crystals. Hardy *et al.* (20) mapped the locus that determines this phenotype to position 45.7 on the X chromosome, and the region was cloned by Lovett *et al.* (21). Livak (22) analyzed the genomic organization of *Ste* and found that it occurs as a 1250-bp sequence in repeated arrays on the X chromosome, and a related sequence occurs on the Y chromosome with a 2.6- to 3-kilobase repeat. Because the presence of the Y chromosome-linked sequences prevents expression of the Stellate phenotype, the Y chromosome-linked family is also called *Su(Ste)*. Rough estimates using the Oregon-R strain indicate about 200 copies on the X chromosome and at least 80 copies on the Y chromosome (22). Low copy number X chromosomes are correlated with appearance of needle-shaped crystals in primary spermatocytes of X0 males, whereas high copy numbers (such as in Oregon-R) are

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Abbreviations: rDNA, genes for rRNA; NO, nucleolus organizer.

associated with star-shaped crystals. Phenomena analogous to compensation and magnification have not been investigated for *Ste*.

Pairing of the sex chromosomes in *Drosophila* is accomplished by heterochromatic regions called collochores, located in close proximity to the nucleolus organizers (NO) in heterochromatin on the X chromosome and on the short arm of the Y chromosome (23). Whether the NO regions in fact are the collochores is suggested by the meiotic instability of sex chromosomes in NO^- flies, such as sc^4-sc^8 (24, 25). Whether the variation in copy number of rDNA in natural populations is associated with the integrity of segregation is of clear importance to the evolution of copy number.

In this report we estimate the number of copies of rDNA and *Ste* in a series of Y chromosome replacement lines and consider the functional significance of the variation by comparing these estimates to segregation behavior of the Y chromosomes.

MATERIALS AND METHODS

Origin of *Drosophila* Lines. Twenty-five lines were started from single females trapped in central Pennsylvania, and 9 lines were of diverse geographic origin. Replacement backcrossing was used to produce Y chromosome replacement lines, bearing only a single Y chromosome from each original isofemale line in a constant genetic background (26). Males of the replacement lines were crossed to virgin females bearing the $Df(1)bb^{l\text{-}158}$ *y* chromosome (from the Pasadena stock center) and yellow male offspring, bearing the $bb^{l\text{-}158}$ and the Y chromosome from the replacement line were used for DNA extractions. Because adults were used for all extractions, estimated copy numbers reflect germ-line differences to the extent that the relative levels of polytenization of *Adh* and rDNA do not vary across lines.

Genomic DNA Extraction. Total genomic DNA was isolated from the 34 lines following the protocol of Clark and Lyckegaard (27). RNA was removed by thorough RNase digestion.

DNA Slot-Blot Analysis. The Bio-Dot SF slot-blot apparatus was used to focus the genomic DNA in a thin line on Zeta-Probe blotting membranes (Bio-Rad). The DNA samples were denatured in 0.4 M NaOH for 10 min and neutralized by addition of an equal volume of 2 M NH_4OAc (pH 7). The denatured DNA was applied in a randomized-block pattern on 48 slots per membrane, representing DNA from duplicate pairs of each of 24 lines. Each line was tested with a minimum of eight replicates distributed on four membranes. Briefly 400 μl of 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) was added to each well after the samples had filtered through and a vacuum was applied until the sample wells were completely dry. The membrane was rinsed in 2× SSC, air-dried, and baked at 80°C for 1 hr prior to hybridization.

Plasmid DNA. The membranes were hybridized with three plasmids. The first plasmid, p13E3, containing the *D. melanogaster Adh* gene (alcohol dehydrogenase, EC 1.1.1.1) in a 4.75-kb *Eco*RI fragment cloned into pUC13, served as a single-copy control for quantifying the total amounts of DNA bound to the membranes. The second plasmid, pDmr.a51#1, contains a complete 11.5-kilobase intron-negative rDNA repeat from the X chromosome cloned into pACYC184. The third plasmid, pSX1.3, is derived from pSP64 and contains a 1269-bp *Xba* I *Ste* gene insert. The plasmids were labeled with [α-^{32}P]dCTP by nick-translation (28) prior to hybridization.

Hybridization. The membranes were prehybridized at 65°C for 10 min with agitation in a prewarmed mixture of 1% bovine serum albumin/1 mM EDTA/7% (wt/vol) $NaDodSO_4$/0.5 M sodium phosphate, pH 7.2. They were never allowed to dry completely after the first prehybridization. The prehybridization solution was removed and replaced with the same solution and the denatured probe DNA. The hybridization continued at 65°C for 18 hr with agitation. To remove nonspecifically bound probe after the hybridization, the membrane was washed at room temperature for 15-min periods in 2× SSC/0.1% $NaDodSO_4$, 0.5× SSC/0.1% $NaDodSO_4$, and 0.1× SSC/0.1% $NaDodSO_4$ sequentially. The radiographic exposure was made with the moist membrane enclosed in a sealed plastic bag. A series of exposures was made for each hybridization, and the intensities of the bands on the resulting autoradiographs were quantified by computing the peak areas with scanning laser densitometry (LKB Ultroscan XL). Before each new hybridization the previously used probe was removed by washing the membrane in 0.4 M NaOH at 65°C for 30 min, and then neutralizing with 0.1× SSC/0.5% $NaDodSO_4$/0.2 M Tris·HCl, pH 7.5 at 65°C for 30 min. A 24-hr autoradiographic exposure was then done to verify the complete removal of the labeled probe. Subsequent probes were hybridized and assayed as described above.

Statistical Analysis. Each autoradiograph had bands that spanned beyond the linear range of the film, so two types of analysis were done. The first restricted attention to exposures in the linear range, and the second made use of all of the data by fitting the exposures to the full sensitometric curve of the film. This was done by doing a logistic transformation, $D_{ijk} = \ln[p_{ijk}/(1 - p_{ijk})]$, where p_{ijk} and D_{ijk}, respectively, are the scaled and transformed band density of replicate k, exposure j, line i. The following model was then fitted by least squares:

$$Q = \sum_i \sum_j \sum_k \{D_{ijk} - [\beta_1 \log(t_j) + \alpha_{ik}]\}^2,$$

where β_1 is a slope parameter for the sensitometric curve of the film common to all lines and replicates, t_j is the exposure time, and α_{ik} is the intercept estimated separately for each replicate of each line. The estimates of β_1 and α_{ik} that minimize Q were obtained numerically using a simplex algorithm (29). The utility of this method was checked by blotting a standard series of six replicates of eight known DNA concentrations and exposing the autoradiographs for six different periods of time.

RESULTS

Standards and Model Verification. From the band densities of the series of standards, least-squares estimates of the time necessary for each sample to attain half saturation of the film were determined. The reciprocals of these times on a logarithmic scale are inversely proportional to the amounts of DNA on the membrane. The fit to the logistic model is presented in Fig. 1, along with a plot showing the correspondence between true and estimated quantities of DNA. The correlation between the true and estimated values is 0.954.

rDNA Copy Number Estimates. Copy number variation is apparent from the slot-blot autoradiographs, because there was greater variation in density of the rDNA probe signal (Fig. 2*B*) than there was from the single copy *Adh* gene probe (Fig. 2*A*). Densitometric scans of multiple exposures of these autoradiographs were used to estimate copy number both by the regression method given above and by taking the ratios of rDNA to *Adh* gene band densities using only exposures in the linear portion of the sensitometry curves. The line means of relative copy numbers estimated by these two methods were highly correlated ($r = 0.956$), but, because the regression method used more of the data and yielded smaller standard errors, only the regression estimates are reported in Fig. 3*A*. The 6-fold range in rDNA copy numbers is consistent with the striking variation in band density seen in Fig. 2*B*. There

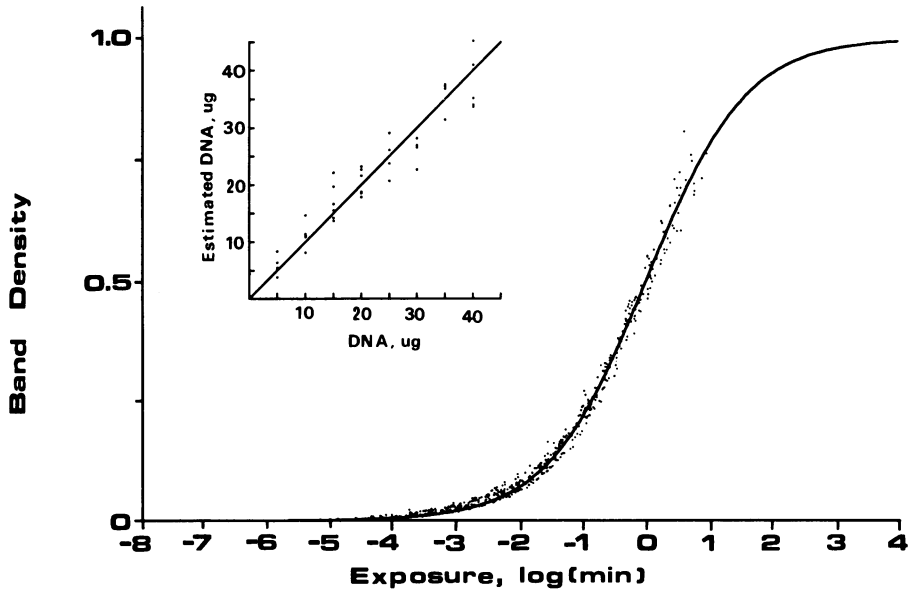

FIG. 1. Least-squares fit of the standard test series to the logistic sensitometric curve of the autoradiographic film. (*Inset*) Plot of estimated μg of DNA against actual amount loaded to the slots of the standard series.

was no evidence for population structuring with respect to copy number, as the 25 lines from Pennsylvania span the range in copy numbers of the geographically diverse lines.

***Ste* Gene Copy Number Estimates.** Separate blots of females bearing two copies of the X chromosome gave a measure of the relative hybridization efficiency of the *Ste* probe to X chromosome- and Y chromosome-linked copies of *Ste*.

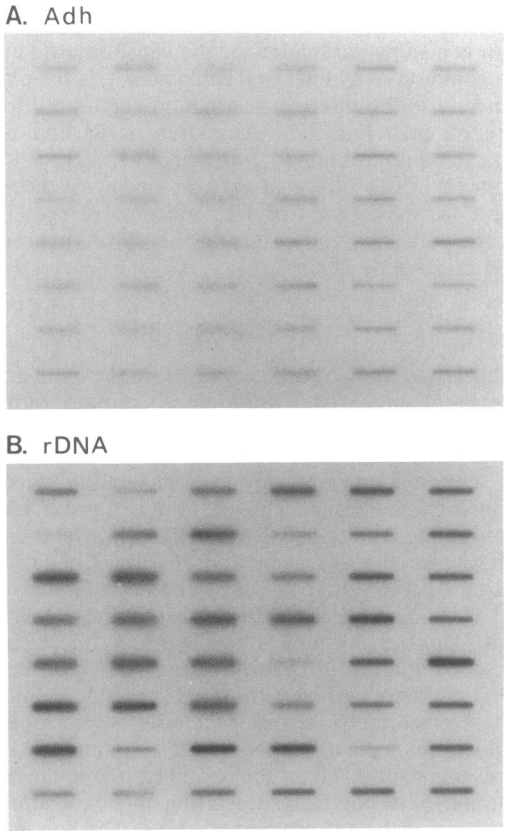

FIG. 2. Autoradiographs of slot blots probed with *Adh* (*A*) and the same membrane washed and reprobed with rDNA (*B*). The approximate evenness of exposure seen in *A* indicates the degree of constancy of slot loading, since *Adh* is present only once per haploid genome in all of these lines. The variation in density in *B* reflects differences in rDNA contents.

Subtracting half of this amount from the male data allows estimates of Y chromosome-linked *Ste* copy number. The multiplicity of copies of *Ste* varied over a 3-fold range (Fig. 3*B*).

Gene Copy Number and Segregation Effects. The possible influence of rDNA copy number on sex chromosome segregation was tested by correlation, using previously reported segregation values (26). The correlation coefficient for the values of the line means of rDNA copy number and sex chromosome segregation was 0.039 (not significant) and for *Ste* copy number and segregation the correlation was 0.035 (not significant). The lack of correlation with *Ste* copy number further confirms the proposal that *Ste* has little if any role in chromosome pairing, since X chromosomes lacking rDNA but having multiple *Ste* copies fail to pair. Whether gene copy number influences the variance in segregation rather than the mean was also tested by correlations of copy number on the standard errors of segregation (for rDNA, $r = 0.176$; for *Ste*, $r = 0.040$). Since none of these correlations approached significance, we conclude that there is not sufficient copy number variation in these population samples to have an influence on segregation. This does not indicate whether an important function of rDNA on the Y chromosome is related to meiotic stability of the sex chromosomes, because lower copy numbers may be necessary to reveal aberrant segregation.

DISCUSSION

This study was designed to quantify the variation in gene copy numbers on the *D. melanogaster* Y chromosome by using many independent lines, extensive replication for statistical precision (2880 bands were quantified), and a regression method that minimizes the variance of estimates. The high (6-fold) variation in rDNA content of the Y chromosome is not entirely without precedent. Gandhi *et al.* (30) observed a range from 57 to 216 copies among five ethyl methanesulfonate-induced Y chromosome-linked *bb* mutants, and variation among laboratory stocks in X chromosome-linked rRNA gene copy number has been known for some time (31). rRNA gene copy number in maize varies from 5000 to 23,000, and there is no correlation between gene content and cellular rRNA content (32). In *Vicia faba* the rDNA copy number varies more than 95-fold within a

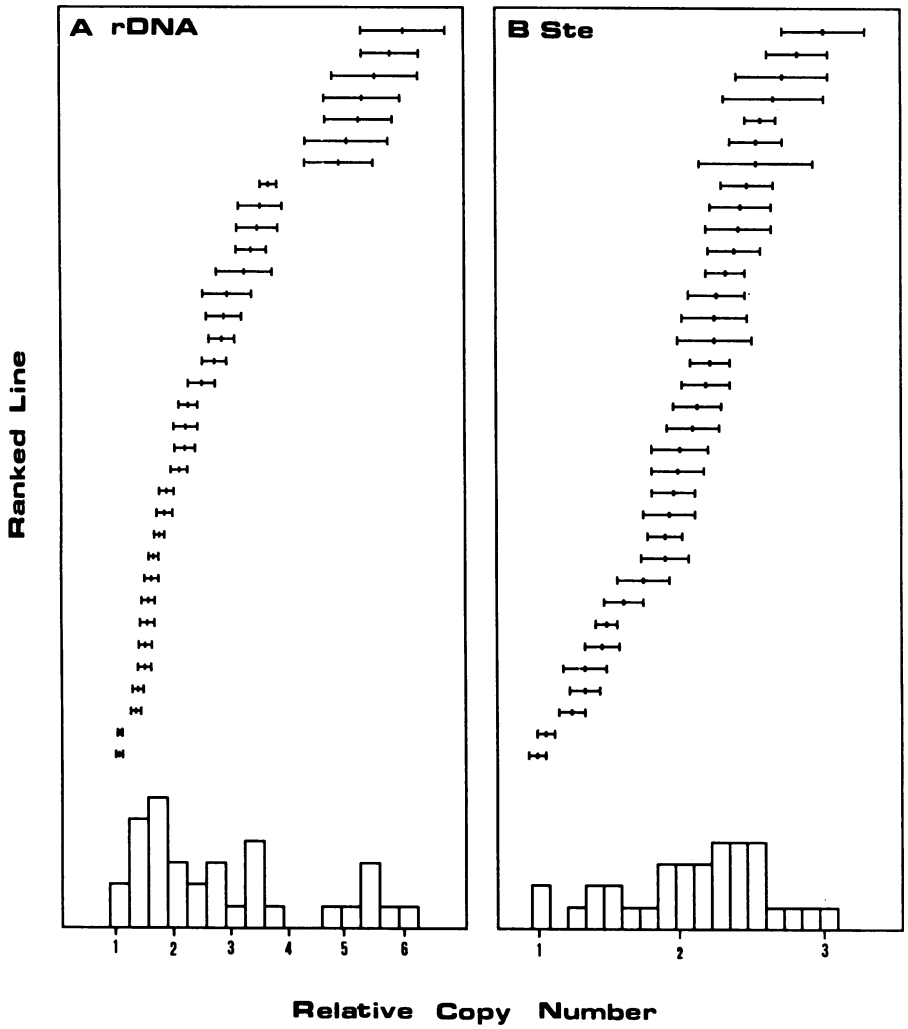

FIG. 3. Relative copy numbers (mean ± SEM) of rDNA and *Ste*, plotted by ranked line means. The lower part of each figure is a histogram showing the frequency distribution of relative gene copy numbers scaled relative to the line with the lowest copy number.

population, whereas within individuals there is a 12-fold range of gene dosage (33). Explanation for these ranges in copy number requires consideration of a diversity of mechanisms that can alter gene copy number.

The population genetic theory of copy number is directly tied to the forces that influence sequence divergence, including mutation, unequal homologous crossing-over, unequal sister chromatid exchange, nonhomologous exchange, gene conversion, and random drift (34). Not only the magnitudes of many of these forces but also the strength and nature of selection are influenced by copy number, because tolerance of altered expression is likely to be greater with higher copy numbers. Although unequal exchange events serve to homogenize sequences, they are also a source of variation in copy number. Drift and selection are the primary forces that can reduce the variance in copy number, so the observed variance in copy number is indicative of the relative rates of homogenization and generation of length variants. Models that exclusively invoke natural selection fail to maintain Y chromosome-linked polymorphism (35), underscoring the necessity to consider drift and the various rates of exchange. Models that invoke only unequal exchange can generate the observed patterns of spacer length variation, but only with restrictive conditions on parameters (36). The high variance in Y chromosome-linked copy number appears contrary to the expectation based on a pure drift model, since the effective population size of the Y chromosome is smaller than that of the X chromosome (37). An unresolved theoretical problem is the simultaneous maintenance of high levels of variability in both copy number and sequence, since an unequal exchange inflates copy number variance but homogenizes the sequences of individual copies.

Experimental observations show that the Y chromosome-linked rDNAs have longer intergenic sequences than do X chromosome-linked copies (38), and more than half of the X chromosome repeats have insertions that are never on the Y chromosome (10, 11), and, despite the extensive similarities in restriction maps (39), there are diagnostic sequence differences in the 18S gene (40). A survey of restriction-site variation revealed that the Y chromosome had greater interpopulation variation in sequence than did X chromosome-linked sequences (41). These observations clearly show that the rate of intrachromosomal exchange is greater than the rate of interchromosomal exchange. Experiments that directly recover unequal exchange events yield estimates in the range of 10^{-4} to 10^{-5} for the rate of unequal crossing-over within rDNA (42, 43). When highly homozygous *Drosophila* were artificially selected for high and low abdominal bristle counts, response was shown to be at least partially mediated by the rDNA array (44). Response was greater in females than males, corresponding to observed changes in X chromosome-linked but not Y chromosome-linked rDNA copy number. Selection experiments yielded an estimated rate of unequal exchange of 3×10^{-4} per gamete generation, and a definitive X–Y chromosome exchange product was recovered in the form of a compound X–Y chromosomal translo-

cation (38). Rates of unequal exchange are apparently great enough to result in concerted evolution, homogenizing sequences on a chromosome (45), and this might generate and maintain copy number variation as well.

Magnification, the term applied to germ-line increases in rDNA number, can occur as either a single large jump in copy number or gradually over several generations (31, 46, 47). There remain uncertainties about the details of the mechanism of magnification, but the failure of ring X chromosomes to magnify strongly implicates the involvement of unequal sister chromatid exchange (48, 49). The tendency for only low copy number chromosomes to magnify has important implications for the evolution of rRNA gene copy number, since it suggests a self-regulating mechanism whose theoretical consequences have not been explored. Finally, transposition may be relevant to the regulation of the proportion of rDNA repeats that bear inserts, since the type II insert in *Bombyx* rDNA bears sequence similarity to retroposons (50).

A potentially important factor in the evolution of copy number is the degree of functional constraint on the multigene families. Y chromosomes lacking *Ste* sequences result in male sterility, so there is clearly a lower bound on the number of copies of this gene compatible with transmission. There is a good correspondence between the number of insertion-free (transcriptionally active) rDNA repeats and the degree of the bb phenotype (51). There is a poor correspondence between the magnitude of phenotypic effects and gene copy number, suggesting that there is underlying variation in the proportion of nonfunctional genes (30). In any case, there are many more copies of rDNA than are necessary for pairing, since *bbl* chromosomes exhibit normal meiotic behavior. Our results show that despite the magnitude of Y chromosome copy number variation, there is no influence on segregation of the sex chromosomes. Nevertheless, the influence of aberrant segregation may be a factor preventing the complete loss of rDNA from the Y chromosome and provides an evolutionary constraint that is distinct from transcriptional activity.

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