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Expression of *I-Cre*I Endonuclease Generates Deletions Within the *rDNA* of Drosophila

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ABSTRACT

The *rDNA* arrays in Drosophila contain the *cis*-acting nucleolus organizer regions responsible for forming the nucleolus and the genes for the *28S*, *18S*, and *5.8S/2S* RNA components of the ribosomes and so serve a central role in protein synthesis. Mutations or alterations that affect the nucleolus organizer region have pleiotropic effects on genome regulation and development and may play a role in genomewide phenomena such as aging and cancer. We demonstrate a method to create an allelic series of graded deletions in the Drosophila *Y*-linked *rDNA* of otherwise isogenic chromosomes, quantify the size of the deletions using real-time PCR, and monitor magnification of the *rDNA* arrays as their functions are restored. We use this series to define the thresholds of *Y*-linked *rDNA* required for sufficient protein translation, as well as establish the rate of *Y*-linked *rDNA* magnification in Drosophila. Finally, we show that *I-Cre*I expression can revert *rDNA* deletion phenotypes, suggesting that double-strand breaks are sufficient to induce *rDNA* magnification.

THE genes that encode three of the four RNA components of ribosomes, the *28S* (sometimes called *26S*), *18S*, and *5.8/2S* rRNAs, are found in repeated arrays of cistrons on the *X* and *Y* chromosomes of *Drosophila melanogaster* (Wellauer and Dawid 1977; Tautz *et al.* 1988). Each ribosomal RNA gene array contains ~100–300 copies of the multigene cistron (Tartof 1973; Long and Dawid 1980), although the number may vary within laboratory or wild strains (Lyckegaard and Clark 1989; Clark *et al.* 1991; Averbeck and Eickbush 2005). The individual cistrons within an array, as well as the arrays on the two sex chromosomes, are redundant, since only a subset are required to supply the demands of normal protein synthesis (Ritossa and Atwood 1966; Gersh 1968). Deletions within the arrays are without phenotype unless extreme enough to limit the total number of copies of cistron in the cell to < ~200 (Ritossa 1968; Tartof 1973).

The *rDNA* arrays are volatile, as the number of cistrons per array varies from generation to generation (Ritossa 1968; Tartof 1974) or within the cells of an individual (Cohen *et al.* 2003, 2005). Wild-type arrays are seen to reduce through somatic development and deleted *X*-linked arrays to magnify during mitosis and meiosis (de Cicco and Glover 1983; Hawley and Tartof 1985), retaining an average of ~200 copies in each array. Measurements of array sizes have been accomplished genetically (*e.g.*, Marcus *et al.* 1986), which has the benefit of revealing individual cell information, but at low resolution and only in specific tissues, or molecularly by using membrane hybridization analyses, which necessitate the averaging of array size over many individuals or many tissue types (Tartof 1973; Lyckegaard and Clark 1989).

The *rDNA* array is arguably the best-understood locus controlled by epigenetic regulation (McStay and Grummt 2008) and the best-characterized repeated gene locus, yet many aspects of its size, structure, and regulation have been beyond experimental manipulation. Many studies have sought to investigate the biology of the *rDNA* loci but, with few exceptions (Robbins 1981, 1996), most have suffered from an inability to cause specific, graded, and easily induced damage to the locus. To probe *rDNA* biology, we developed a facile and reproducible system for *rDNA* cistron deletion. The *I-CreI* homing endonuclease cleaves a degenerate consensus, which appears in both Chlamydomonas and Drosophila *rDNA* (Seligman *et al.* 1997; Maggert and Golic 2005).

In this report, we demonstrate that deletions within the *rDNA* arrays can be induced by exposure to *I-Cre*I. We used genetic tests to initially identify deletions to a length less than that necessary to serve as the sole source of rRNA in the cell. We developed a reliable real-time polymerase chain reaction assay to quantify the amount of *rDNA* on these chromosomes, establishing an allelic series of otherwise isogenic *Y* chromosomes. Using this series, we defined thresholds of *Y*-linked *rDNA* array size required for protein synthetic demands. Despite being kept as stocks with wild-type *X*-linked *rDNA* arrays, these *Y*-linked arrays magnified in size. A second exposure to

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*I-Cre*I induced large magnifications that rapidly restored deleted *rDNA* arrays. Our work establishes methods for generating and characterizing mutations of the *rDNA* and expands our understanding of *rDNA* magnification of *Y*-linked *rDNA* arrays.

MATERIALS AND METHODS

Fly stocks and husbandry: The *Y10A* chromosome is $y^+ Yw^+$, *Dp(1;Y)* y^+, *P{w⁺=RSw}10A* (Maggert and Golic 2005). The first exon of the *white*⁺ gene in *RSw* is flanked by *FRT* sequences (Golic and Golic 1996). A chromosome with *FLP*-induced loss of *white*⁺ is referred to as *Y10B*. Prior to using either *Y10A* or *Y10B* for these experiments, we crossed single males to females for three generations prior to our experiments. The *X* chromosome is y^1 w^{67c23}. The *I-Cre*I-expressing line is *P{v⁺ᵗ¹·⁸=hs-I-CreI.R}2A*, v^1/Y; *Sb/TM6b, Ubx* (Maggert *et al.* 2008), obtained from the Bloomington *Drosophila* Stock Center. The attached-*X* chromosome is *C(1)DX*, y^1 f^1 bb^0 (Lindsley and Zimm 1992). Flies were raised on cornmeal molasses agar at 25° and 80% humidity.

Induction and screen for deletions: Flies were allowed to lay eggs for 2–3 days, and larvae to develop for 1 more day. Second and third instar larvae were heat-shocked in circulating water baths at 36°. In experiments involving *Y10A*, larvae were heat-shocked on 2 successive days, each treatment lasting 45 min. In experiments involving *Y10B*, larvae were heat-shocked on 1 day for 45 min.

Heat-shock-induced expression was monitored by under-representation of *I-Cre*I bearing male progeny in relation to *P{v⁺ᵗ¹·⁸=hs-I-CreI.R}2A*, v^1/y^1 w^{67c23} siblings and by cuticle or eye defects indicating expression-induced cell lethality (Maggert and Golic 2005). *X–Y* translocation chromosomes were identified as sterile yellow males and yellow+ females and were excluded from analysis.

Real-time polymerase chain reaction: Primers AGCCTG AGAAACGGCTACCA and AGCTGGGAGTGGGTAATTTACG amplify 63 nucleotides of the *18S* gene in the *35S rDNA*. After confirming single melting curve kinetics using an ABI Step-One real-time polymerase chain reaction machine (Applied Biosystems) running Step-One v1.0 software, we used the Power SYBR Green master mix (Applied Biosystems) reagent, 500 nm primers, and 10 ng nucleic acid with 40 cycles alternating between 95° for 3 sec and 60° for 30 sec. DNA samples were prepared using a modified procedure from K. Dobie (Gloor *et al.* 1993; Dobie *et al.* 2001). The organic extractions were followed with ether extraction, rather than ethanol precipitation, which produced 1–2 μg total nucleic acid/fly. Amplification data were processed by determining the point at which fluorescence first crossed a threshold of 10 standard deviations above the average of all previous cycles ("no amplification") of fluorescence from each extract, as determined by the Step-One software. Extracts were run in triplicate (occasionally quadruplicate) identical samples. Samples in discordance with the other samples (a threshold cycle with a difference of >2 standard errors of the mean) were interpreted as errors in reaction or reaction preparation and were excluded. Fewer than fifty of ~5000 total samples were discarded using this criterion. *tRNA*ᴷ·ᶜᵀᵀ genes were amplified using primers CTAGCTCAGTCGGTAGAGCATGA and CCAACGTGGGGCT CGAAC to generate a 63-nucleotide product. Cycle differences between *rDNA* and *tRNA* genes ("ΔC_T") were compared to the same measurement from DNA pooled from a large population (~200) of adult flies or larvae bearing chromosome *Y10B* ("$\Delta\Delta C_T$"), generating the percentage of wild-type *rDNA* quantity. Adult DNA was used for *rDNA*ᵇᵇ lines, and larval DNA was used for *rDNA*ᵇᵇ·¹ lines. The same pooled *Y10B* preparations of DNA were used for all experiments.

We present either standard deviation (with pooled root-sum errors) if individuals are compared to other individuals or standard errors of the mean (with pooled root-squared-sum errors) if array size from individuals is shown.

Cytology and photography: Photographs of adult flies were taken using a Nikon D2H camera attached to a Nikon SMZ-1500 microscope. Neuroblast spreads were prepared following the protocol of S. Pimpinelli, S. Bonaccorsi, L. Fanti, and M. Gatti (Sullivan *et al.* 2000).

RESULTS

Creation of *rDNA* deletions using *I-Cre*I expression: To create an allelic series of deletions within the *rDNA*, we devised an easily employed system to induce varying degrees of damage to isogenic target chromosomes. We chose to make a deletion series of the *Y* chromosome because of the ease with which this chromosome is manipulated in Drosophila (Bridges 1916). Since the *X*-linked *rDNA* array has been the primary target for previous analysis of *rDNA* mutation (summarized in Lindsley and Zimm 1992; Ashburner *et al.* 2005), information on the *Y*-linked *rDNA* would produce the additional benefit of revealing novel information or supporting the generality of the work on the *X*-linked array.

Variability in *rDNA* array size likely exists even within populations derived from a common ancestor (Averbeck and Eickbush 2005). We suspected that small deletions would reveal little useful information; however, larger deletions had the potential to reveal some features of *rDNA* biology, including transcriptional regulation and magnification. Hence, we sought to generate deletions that removed enough of the *rDNA* cistrons to manifest a phenotype.

In the first experiment, we used *Y* chromosome *Y10A*, which contains an active *white*⁺ transgene near the telomere of the short arm (Golic *et al.* 1998), and a translocation between the *X* and the tip of the long arm to introduce a *yellow*⁺ marker. For the second experiment, we used a related *Y* chromosome, *Y10B*, which differs only in the absence of the promoter and first exon of the *white*⁺ gene, rendering it *white*⁻. We generically refer to either chromosome as *Y10*, meaning either *Y10A* or *Y10B*.

We crossed heat-shocked *P{v⁺ᵗ¹·⁸=hs-I-CreI.R}2A*, v^1/*Y10* males *en masse* to virgin y^1 w^{67c23} females (Figure 1, generation 1) and collected male offspring. Individual male progeny were each crossed separately. The number of translocation chromosomes (Table 1) confirmed that *I-Cre*I was expressed and that damage occurred to the *X*- and *Y*-linked *rDNA* arrays and was subsequently repaired. We reasoned that, within the chromosomes that we collected by isolating individual male progeny, we would find an allelic series of *rDNA* deletions.

A genetic test for *rDNA* deletion: Since the *I-Cre*I-mediated damage to the *Y* chromosomes is specific to

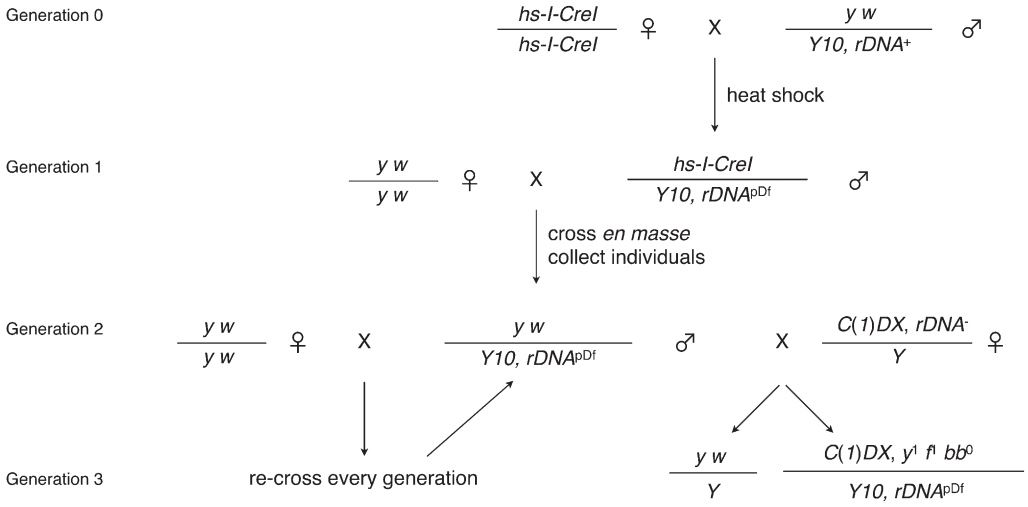

FIGURE 1.—Genetic cross and screen for *Y, rDNA* deletions. In generation 0, females harboring a heat-shock-inducible *I-Cre*I nuclease are mated to males with a recently isogenized *yellow*$^+$-marked *Y* chromosome. Males were heat-shocked as larvae and crossed to a common *yellow white* stock en masse in generation 1. Collecting individual males in generation 2 allowed us to sample independent *I-Cre*I-induced *rDNA* events of the *Y* chromosomes. Males were crossed to both fresh *yellow white* females to establish a stock and to *C(1)DX* females, whose compound-*X* chromosome lacks *rDNA*, to determine if damage to the *rDNA* had occurred. Damage could be assessed as an altered female-to-male ratio (at an extreme, 0:1) or as *bobbed* female phenotypes (in generation 3). In every subsequent generation, males were backcrossed to the maternal genotype (*yellow white*) to maintain the stock and *C(1)DX* females to retest the *rDNA* array. Genetic nomenclature: *hs-I-Cre*I is *P{v*$^{+t1.8}$*=hs-I-CreI.R}2A*, v^1; *y w* is y^1 w^{67c23}; *Y10* is y^+ Yw^+, *Dp(1;Y)* y^+, *P{w*$^+$*=RSw}10A*, or y^+ Yw^-, *Dp(1;Y)* y^+, *P{w*$^-$*=RSw*$^-$*}10B*; and *C(1)DX* is *C(1)DX*, y^1 f^1 bb^0.

the *rDNA* array, we could easily monitor the extent of damage genetically by making the potentially damaged *Y* chromosome the sole source of *rDNA* to the organism. Compound chromosome *C(1)DX*, y^1 f^1 bb^0 (*C(1)DX*) contains no *rDNA* genes (LINDSLEY and ZIMM 1992), and so we replaced the normal *Y* chromosome in a *C(1)DX/Y* stock with the *rDNA potential Deficiency* (*Y10, rDNA*^{pDf}) chromosomes from our study (Figure 1). We expected large deletions to be inviable and moderate deletions to be subviable or express a bobbed phenotype.

We tested 1160 individual *Y10, rDNA*^{pDf} chromosomes using this assay and identified 23 *Y* chromosomes incapable of supplying sufficient rRNA for survival (1.9%), 9 *Y* chromosomes that expressed a majority penetrant (>50% of flies showed the phenotype) bobbed cuticular phenotype (0.7%), and 92 more that exhibited a sex ratio significantly different from unity (7.9%) (see Table 1 for summary). Each potential reduction chromosome was retested; those with no or bobbed female progeny again produced similar female progeny upon retest, but those that showed altered sex ratios showed normal ratios upon retest. Lethality and the bobbed phenotypes were thus reliable indicators of *rDNA* deletion; however, the use of subviability (and consequent sex-ratio distortion) was not.

We wanted to confirm that the lethality phenotypes that we observed were due to reduction of the *rDNA*, so we crossed males from eight of our identified *Y10, rDNA*^{pDf} chromosomes (three from *Y10A* and five from *Y10B*) to females of the genotype *In(1)sc*^{4}*sc*^{8}*/FM7a, B*^{S}. *In(1)sc*^{4}*sc*^{8}, like *C(1)DX*, lacks *rDNA*. Half of the male progeny of the cross were expected to express a Bar phenotype (*FM7a/Y*) and half to be non-Bar [*In(1)sc*^{4}*sc*^{8}*/Y10B, rDNA*^{pDf}], unless the *rDNA* was removed, in which case the non-Bar class of males would be absent or express a bobbed phenotype. We found strict concordance between the *C(1)DX/Y10, rDNA*^{pDf} lethality or bobbed phenotypes and the *In(1)sc*^{4}*sc*^{8}*/Y10B, rDNA*^{pDf} lethality or bobbed phenotypes (data not shown), indicating that the lethality is linked to the *Y* chromosome and most likely due to *rDNA* deletion, and not to the induction of other genomic alterations that interacted with the *C(1)DX* background to produce lethality.

TABLE 1

Derived *Y,rDNA*^{Df} chromosomes

Screen	Parental Y chromosome	Chromosomes screened	Altered sex ratio	*T(X;Y)*	*Y,rDNA*^{bb}	*Y,rDNA*^{l}
1	*Y10A*	560	32	12	0	7
2	*Y10B*	600	60	6	9	16
Total		1160	92	18	9	23

*I-Cre*I-induced deletions within the *rDNA* of two related *Y* chromosomes. *Y10A* and *Y10B* differ only by a *white*$^+$ transgene. Progeny that displayed altered sex ratios and translocation chromosomes [*T(X;Y)*] were not pursued. Deficiencies of the *rDNA* (*Y,rDNA*^{bb} and *Y,rDNA*^{l}) are described in the text.

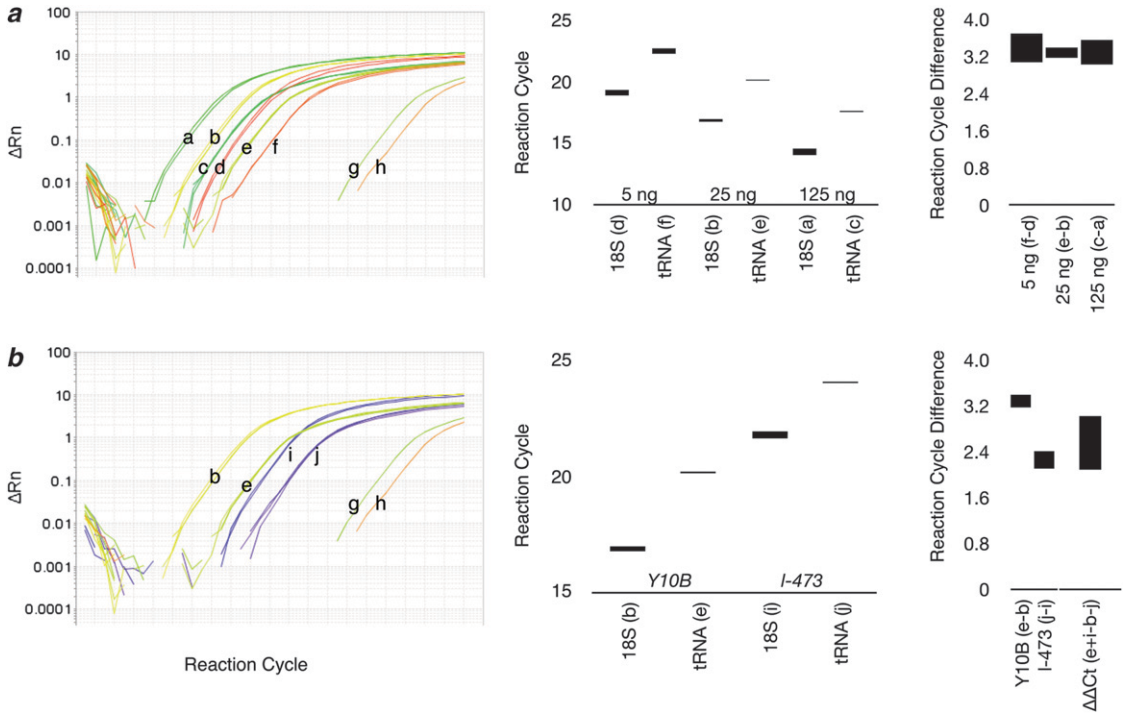

Figure 2.—Real-time qPCR to measure *rDNA* content. (a) Traces of qPCR reactions to amplify the *18S* rRNA and tRNA^{K} genes at three different concentrations of DNA extracted from *C(1)DX/Y10B* females. Traces show triplicate reactions set in parallel; each triplet is labeled with a letter (a–f), which corresponds to the data in the accompanying graphs. g and h are no-template controls. Bar graphs show the average ± standard error of the mean ranges for amplification cycles (Ct). The rightmost graph shows the difference between *rDNA* and *tDNA* threshold cycles (ΔCt) with ranges equal to root pooled squares of standard errors. (b) Traces of qPCR reactions to amplify *18S* and tRNA^{K} genes from wild type (*Y10B*, traces b and e) and a *rDNA* deficiency chromosome (*l-473*, traces i and j). b, e, g, and h are the same traces as in a. Accompanying graphs show Ct for these reactions: the rightmost graph shows ΔCt as a measure of the *rDNA/tDNA* copy number, and ΔΔCt shows the difference between *l-473* and *Y10B* in Ct, corrected (by the *tDNA* measurement) for DNA concentration. The difference in *rDNA* copy number is $2^{\Delta\Delta Ct}$. The *y*-axes are either cycles of qPCR or differences in cycles between different samples.

We confirmed that the only cytologically visible alteration to the chromosome structures was in *Y*-linked band *h20*, the location of the *rDNA* locus (data not shown).

Since we interpreted our results to mean that all of the identified lines possessed significant reduction of the *rDNA*, these chromosomes from hereon will be referred to as *Y10B, rDNA*^{l} or *Y10B, rDNA*^{bb} (or, generically, *Y10B, rDNA*^{Df}), consistent with established nomenclature for *bobbed-lethal* or *bobbed* alleles with reduced *rDNA* copy number.

Molecular test and quantification of *rDNA*: The genetic test for *rDNA* array size relies on active *rDNA* cistrons. We sought a method to quantify the size of the deletions irrespective of genetic activity and so developed a real-time (quantitative) polymerase chain reaction (qPCR) to measure the copy number of *rDNA* template.

To normalize the *rDNA* qPCR amplification rate, we chose a denominator that fulfilled several criteria. First, we needed a normalizing DNA sequence with a high copy number, which would make the quantification of *rDNA* robust despite fluctuations in DNA yield from individual flies. Second, we needed a sequence that did not vary between individuals within a population or between strains so that our results would be easily comparable without performing cumbersome crosses to establish isogeny. Third, since many tandem-repeat (arrayed) DNAs are eliminated during development (Cohen *et al.* 2003, 2005), we wanted to find a dispersed repeat. For these reasons, we chose the high-copy-number *tRNA*$^{K\text{-}CTT}$. Although lysine is encoded by three anticodons, the CTT isotype is most common, and all 15 *tRNA* genes of this isotype (in a haploid genome) have identical sequence (Schattner *et al.* 2005). Amplification of 30 *tRNA*$^{K\text{-}CTT}$ genes (in a diploid genome) then was used as a denominator in our calculations. We are aware that not all *tRNA* genes may be equally amplified in our reaction, so absolute values of *rDNA* copy number may be inaccurate; however, the copy number of *rDNA* (on *Y10B* or an unrelated unmarked *Y*), by these calculations, is ~290 (Figure 2, 95% confidence interval of 270–315 copies), in agreement with population studies performed by many labs using other techniques (Tartof 1973; Shermoen and Kiefer 1975; Long and Dawid 1980; Lyckegaard and Clark 1989). We are cautious about comparing absolute copy numbers of *rDNA* derived from different techniques and so report quantification relative to our *Y10* chromosomes.

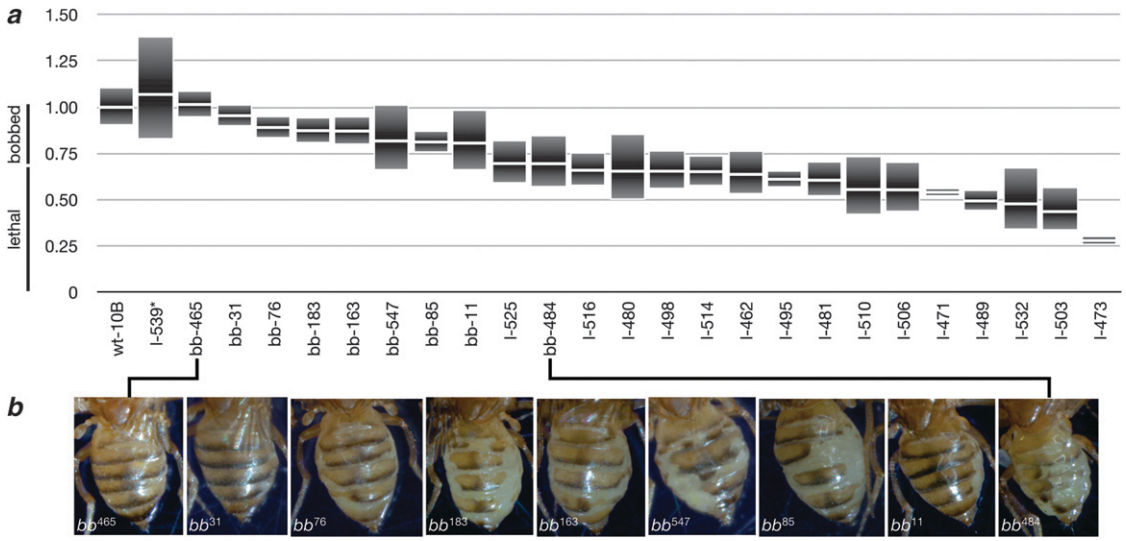

FIGURE 3.—*Y, rDNA*^{Df} allelic series. qPCR was used to measure *rDNA* array size in the alleles generated in this work. (a) 10B is the progenitor chromosome. The remainder are the recovered alleles, sorted by average size. Data are presented as the average ± standard error of the mean. Chromosome names indicate their phenotype (wt, wild-type; bb, bobbed; l, lethal) as the sole source of *rDNA* in the organism, as well as their allele number. The approximate range that corresponds to those phenotypes is indicated at the left of the graph. The *y*-axis is ratio of wild-type *Y10B* chromosome *rDNA* content. (b) Bobbed flies were photographed and are presented in order of decreasing *rDNA* array size (taken from a), which correlates with an increasing severity of the bobbed phenotype.

By titrating the template, we determined that the amount of template is free to vary over a concentration range of at least 25-fold and that the denominator *tRNA*$^{K\text{-}CTT}$ is able to normalize the signal to a relative copy number (Figure 2). We have been able to detect template *rDNA* from as few as five genome equivalents (data not shown), a total template *rDNA* copy number of <2000, and a *tRNA*$^{K\text{-}CTT}$ copy number of ~150; however, our analyses of *Y10B, rDNA*^{Df} presented here were done with 10–20 ng of total nucleic acid to ensure that we were well within the range of linear sensitivity.

To make the reduced *rDNA* array unique within the genome, we crossed y^1 w^{67c23}/*Y10B, rDNA*^{Df} single males to *C(1)DX/Y* females. Female progeny are of two types: *C(1)DX/*y^1 w^{67c23} (triplo-*X*) metafemales that die late in development and are identifiable by their yellow phenotype (LINDSLEY and ZIMM 1992) and *C(1)DX/Y10B, rDNA*^{Df} females that have only *Y*-linked *rDNA* and are identifiable by their yellow+ phenotype. Flies devoid of *rDNA* still possess rRNA by virtue of maternally loaded RNAs and ribosomes and, in our hands, survived to late larvae or early pupae stages. Hence, we were able to purify DNA from *C(1)DX/Y10B, rDNA*^{Df} larval, pupal, or adult females whose only *rDNA* was the *Y*-linked array.

The results of our analyses of all *Y10B, rDNA*^{Df} are presented in Figure 3a, which shows quantification from multiple (three to seven) *C(1)DX/Y10B, rDNA*^{Df} female siblings from single fathers in the second generation after being isolated as independent stocks (Figure 1, generation 3). The ranges shown are pooled standard errors from replicate reactions using DNA from three to eight individuals of generation 2. These ranges include experimental error and standard deviation of the population analyzed, sorted by mean after the reference pool of *Y10B*. We used wild-type (*Y10B*) reference DNA preparations separately for adults and larvae.

As expected, the *rDNA* arrays that are largest among our allelic series express a bobbed phenotype. Figure 3b shows the abdomens of surviving *C(1)DX/Y10B, rDNA*^{Df} females carrying *bobbed* alleles of the *rDNA*. Moreover, we saw a correlation between the deletion size and the expressivity of the bobbed phenotype. More extensive deletion of the *rDNA* caused bobbed-lethal phenotypes. Hence, two transitions are defined by this graph: the *wild-type* to *bobbed* transition, and the *bobbed* to *lethal* transition.

The *wild-type* to *bobbed* transition occurred at ~90% of the hemizygous (*Y*-linked) *rDNA* cistrons, or ~260 copies. Although we expected to define such a transitional *rDNA* size, we were surprised that, in our studies of the *Y* chromosome, this transition was higher than in other studies that investigated the *X*-linked *rDNA bobbed* threshold. The *X rDNA* locus required a deletion to ~50–80% of the wild-type size, or to ~150–200 cistrons, to produce a bobbed phenotype (TARTOF 1973; TERRACOL and PRUD'HOMME 1986). This difference may be due to the disparate chromosomes used in these studies, to differences in the proportion of intact and R1- and R2-interrupted cistrons (LYCKEGAARD and CLARK 1989; AVERBECK and EICKBUSH 2005), or to differences in the quantification techniques used in each study.

The transition from bobbed to lethal occurred in ~65% of the hemizygous (*Y*-linked) level of *rDNA*. This is ~190 copies according to our calculations and is again higher than previous studies that indicated as few as 114

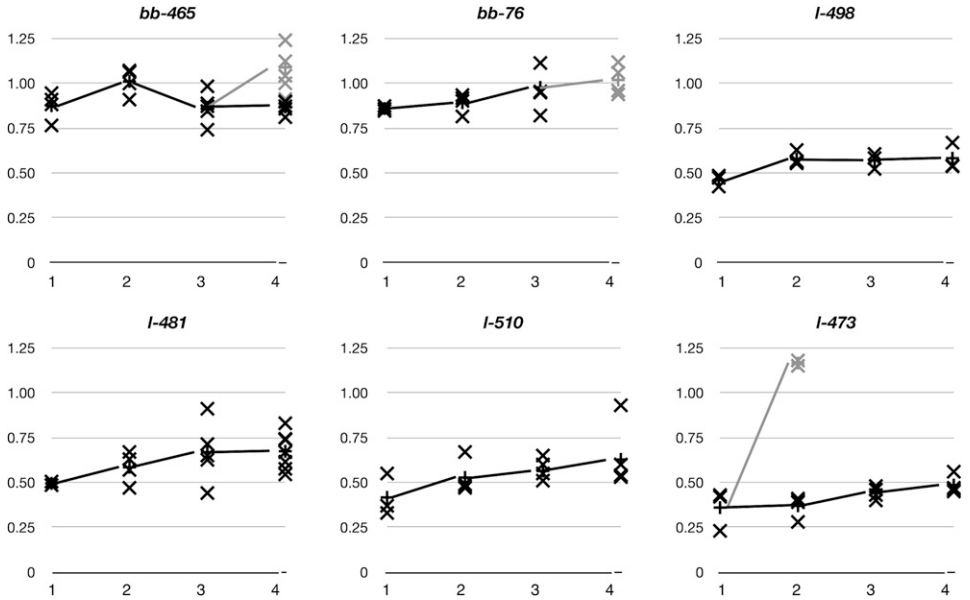

Figure 4.—*rDNA* arrays undergo slow gradual magnification as well as sporadic fast magnification. Six chromosomes were monitored every generation by selecting four to eight individuals for *rDNA* array size measurement. The data for each individual are shown (x's), as well as the average of the population (connected by lines). Solid data points indicate individuals with the same phenotype (lethal or bobbed) as previous generations, and shaded data points are from individuals whose phenotype changed (to wild type for *bb-465* or to bobbed for *l-473*). The *x*-axes are the successive generations after establishment as stock. The *y*-axes are the ratios to wild-type *Y10B* chromosome *rDNA* content. For clarity, standard errors are not depicted.

copies of *X*-linked *rDNA* are sufficient for viability (Terracol and Prud'Homme 1986).

One chromosome, *Y10B, rDNA*$^{l\text{-}539}$, carried a lethal allele of the *rDNA* despite showing an array size larger than *Y10B, rDNA*$^{+}$. We do not know why ample *rDNA* would not supply sufficient rRNA, but consider that the cistrons may have been damaged during *I-Cre*I-induced damage, magnification may have occurred using inactive R1- or R2-interrupted cistrons as template, the copies on the chromosome may be epigenetically inactive, or some other explanation may account for this (Terracol and Prud'Homme 1981; Terracol 1987).

Evidence for *rDNA* magnification: We initially established stocks of three of the seven *rDNA*^{l} deletions derived from *Y10A*, assuming that they would be stable as stocks containing an *X* chromosome with a normal *rDNA* array, since it is generally accepted that magnification of the *Y* requires special circumstances. Instead, we found that, upon retest after seven generations as a stock, two of the stocks had reverted and produced bobbed-viable and wild-type individuals despite the presence of a fully functional *X*-linked array. *X*-linked *rDNA* magnification is a well-characterized phenomenon (Marcus *et al.* 1986), yet we were surprised to see that the new *Y10A*, *rDNA*^{l} chromosomes exhibited this phenotype after so few generations without obvious selection. Leonard Robbins (1981) showed that many deletion alleles of the *rDNA* are stable once generated, while ours are not. Both those alleles and ours rely on creating damage specifically to the *rDNA* arrays, however by different means. Komma and colleagues showed that certain *Y* chromosomes are capable of magnification even when the cell possesses sufficient *rDNA* (Komma and Endow 1986; Komma *et al.* 1993). This feature is not understood but has been shown to reside on some *Y* chromosomes; it is possible that our chromosome possessed this ability prior to being reduced.

We confirmed that the suppression of the lethal phenotypes in *Y10A,rDNA*$^{l\text{-}3\text{-revertant}}$ and *Y10A,rDNA*$^{l\text{-}39\text{-revertant}}$ did not map to the *X* chromosome or large autosomes (data not shown). We interpreted these results to indicate that the *Y*-linked *rDNA* array had increased in size, rather than the stock accumulating modifiers of *rDNA* expression, which has been shown as an alternate means of *rDNA* magnification (Marcus *et al.* 1986).

To observe magnification as it occurred on our chromosomes, we outcrossed males from each *Y* chromosome stock to virgins of a common y^1 w^{67c23} stock to prevent the accumulation of modifiers that could affect *rDNA* expression. At every generation, we also crossed sibling males to *C(1)DX*, y^1 *f* bb^0/*Y* or *C(1)DX*, y^1 *f* bb^0/*Y*, B^S females to genetically assess the status of the *rDNA* array size in female offspring. This is represented by the recursive recross in generation 3 of Figure 1.

We chose to monitor six chromosomes over four generations. For each generation, we extracted DNA and measured *rDNA* content from 4 to 10 pupae or adults, which allowed us to investigate *bobbed* and *bobbed-lethal* lines. The results are presented in Figure 4.

We observed progressive *rDNA* magnification by monitoring individuals every generation. All lines showed an average array size increase every generation. This gradual increase was varied, but was ~5% (5.6% ± 1.4%) of the wild-type *rDNA* array size when averaged for all individuals between the first and fourth generations, or 7.2% ± 5.6% for each generation, excluding those that underwent large increases (*e.g.*, *Y10B*, *rDNA*$^{bb-473}$) or decreases (*e.g.*, generation 2 to generation 3 for *Y10B*, *rDNA*$^{bb-465}$). These results underscore the variation in the *rDNA* magnification amount, but indicate a trend toward pro-

gressively larger arrays. Consistent with this, five of the six chromosomes (all but *Y10B*, $rDNA^{bb-473}$) showed an increasing coefficient of variance (ratio of standard deviation to average) in each generation. Although some individuals showed arrays that dropped in size compared to the previous generation's average, these were less frequent than were instances where the array magnified. This value, the addition of ~15 copies of *rDNA* per generation, is essentially identical to that observed for the *X* chromosome (Tartof 1973).

Magnification is not constant, but may instead increase by small steps and spend some generations remaining steady at average size. Line *Y10B*, $rDNA^{bb\text{-}498}$ is a lethal allele of *bobbed* and shows a slow increase in *rDNA* content. The initial increase was the largest (11.6%), and the rate slowed for the subsequent two generations (0.5% over two generations). Since we expect that magnification affects every chromosome by differing amounts, this may be an artifact of small sample size. With a small sample size, it is possible to select the less-common individuals whose *rDNA* arrays either have decreased in size or have not changed appreciably. Over the individuals scored in the subsequent generation, this would appear as decreasing or stable *rDNA* arrays, despite the majority of the population increasing in size. A trend of magnification is consistent with our results from the other lines that we analyzed, notably *Y10B*, $rDNA^{l\text{-}481}$, *Y10B*, $rDNA^{l\text{-}510}$, and *Y10B*, $rDNA^{l\text{-}473}$, that *rDNA* size varies between generations and an increase in size is more common than a decrease, leading to a gradual and steady increase in the population.

Line *Y10B*, $rDNA^{bb-465}$ magnified to a size that overlapped with wild type, although all flies were *bobbed*. In the subsequent generation, the average size decreased again, but was still within the previously defined *bobbed* range (Figure 3). In the fourth generation, two classes of flies were seen: those that were bobbed and those that had phenotypically reverted to wild type (shaded in Figure 4). As predicted, those that were wild type in appearance had arrays that had increased more than those that remained *bobbed* and were 108% ± 17% of the wild-type quantity of *rDNA*.

Line *Y10B*, $rDNA^{bb\text{-}76}$ was also originally identified as a *bobbed* reduction. For the next two generations, *bb-76* showed the same expressivity of phenotype. In the third generation, however, the *C(1)DX/Y10B-*$rDNA^{bb\text{-}76}$ females were notably less bobbed, and by the following generation, all female progeny had normal cuticles (shaded data points in Figure 4). This corresponds to the generation in which the average array size reached 101% ± 8% of wild-type level, near the transition that we had defined by analyzing the allelic series of initial deficiencies (Figure 3).

We cannot distinguish between somatic pseudomagnification, which our assay measures, and germline magnification, since the germline is a small fraction of the genomes measured in whole animals. However, it is likely that germline magnification contributes since the average array size grows in subsequent generations. This is particularly evident in those cases in which a chromosome (*e.g.*, *Y10B*, $rDNA^{bb\text{-}465}$ and *Y10B*, $rDNA^{bb\text{-}76}$) reverts to *wild type* and all progeny in that stock do so.

Against a backdrop of steady increase, we also saw two large increases, similar to what is observed for the *X*-linked *rDNA* arrays (Hawley and Tartof 1985; Endow and Komma 1986). Line *Y10B*, $rDNA^{l\text{-}473}$ produced two bobbed flies in the second generation, which corresponded to the two *Y* chromosomes that had very large increases in *rDNA* size (shaded data points in Figure 4). This increase (to 117% ± 2%) was more than two times the size of the progenitor *Y* chromosome (36% ± 11%), a dramatic example of a magnification event that cannot be explained simply by a single unequal sister chromatin exchange.

Induction of magnification by *I-Cre*I expression: Our results indicate that double-strand breaks are sufficient to induce reduction in *rDNA* copy number and that natural processes are then able to magnify the arrays toward their original size. It has been proposed that magnification might rely on double-strand breaks, since flies mutated for genes involved in double-strand break repair are unable to magnify their arrays (Marcus *et al.* 1986). We could test this assertion using a second *I-Cre*I.

We crossed three lethal deletions from our allelic series to females carrying an *I-Cre*I transgene and heat-shocked for 1 hr to induce *I-Cre*I expression (Figure 5). Male progeny were crossed en masse to *C(1)DX/Y* females (generation 1 of Figure 5). Progeny of that cross were expected to be solely males, consistent with the phenotype of these *Y10B*, $rDNA^{l}$ chromosomes. If, however, *I-Cre*I expression induced magnification, we expected to obtain revertant females of genotype *C(1)DX/Y10B*, $rDNA^{l\text{-revertant}}$. The results are presented in Table 2.

Sires harboring each of the three tested *I-Cre*I-exposed *Y10B*, $rDNA^{l}$ chromosomes gave female progeny when crossed to *C(1)DX* females. Most of these were severely bobbed, indicating that the *rDNA* arrays were barely sufficient for rRNA demands, although some were bobbed+. Since these *Y10B*, $rDNA^{l}$ were not able to supply sufficient rRNA prior to *I-Cre*I expression, the *rDNA* array sizes must have increased on those chromosomes. To confirm an increase in *rDNA*, we isolated DNA for quantification (Figure 5). Each chromosome contained magnified *rDNA* arrays. For chromosomes *Y10B*, $rDNA^{l-473}$ and *Y10B*, $rDNA^{l-481}$, the amount of magnification varied between individuals and correlated well with the expressivity of the bobbed phenotype. Some revertants of chromosome *Y10B*, $rDNA^{l-481}$ did not show a large magnification. This may be due to this chromosome being on the threshold of lethal to bobbed, so even small magnifications would be uncovered by this assay, or because the *Y10B*, $rDNA^{l-481\text{-revertant}}$ chromosomes possess a different active-to-inactive ratio of *rDNA*

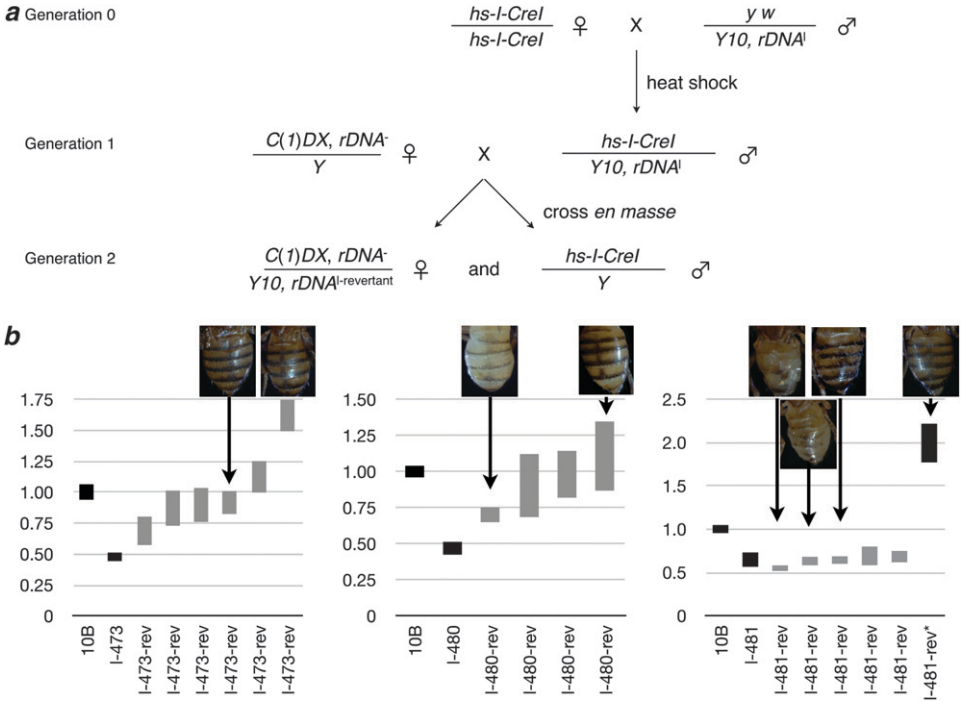

FIGURE 5.—*rDNA* arrays undergo magnification when exposed to *I-Cre*I. (a) *Y* chromosomes with previously reduced *rDNA* arrays were exposed to *I-Cre*I induced by heat shock. This cross is similar to the one described in Figure 1, but here we screened for reversion of the lethal-bobbed phenotype to the bobbed or wild-type phenotype. A control cross was performed in parallel with *X* chromosomes without the *I-Cre*I-expressing transgene. (b) Results of *rDNA* quantification. Each graph contains data showing the relative average for *Y10B* (defined as 1.00) and the parental chromosome prior to heat-shock induction of *I-Cre*I, both as solid data points. Shaded data points are confidence intervals for individuals (the average of replicate qPCR reactions with standard errors of the mean), and photographs of a subset of those individuals show the bobbed phenotype. The final data point, l-481-rev*, is from the control cross, which did not express *I-Cre*I. The large amount of *rDNA* is most consistent with nondisjunction producing a *C(1)DX/Y10B*, $rDNA^{Df}/Y$, $rDNA^{+}$ individual.

cistrons than does the original *Y10B*, $rDNA^{l-481}$ chromosome (TERRACOL and PRUD'HOMME 1981, 1987; TERRACOL 1987; ASHBURNER *et al.* 2005).

Nondisjunction in the *C(1)DX/Y* mothers would also produce flies that appeared as revertants that had magnified to bobbed+, since progeny would be *C(1)DX/Y10B*, $rDNA^{l}/Y$. To identify those events in our analysis, we performed the same cross and heat shock with males of genotype *y w/Y10B*, $rDNA^{l}$. Some sires of genotype *y w/Y10B*, $rDNA^{l}$ did give female progeny, but at a much lower rate than did *I-Cre*I-expressing sires. Surviving females were bobbed+, indicating that they were nondisjunctional progeny. Consistent with this, those surviving females had *rDNA* arrays that were measured to be ~200% of the *Y10B* array (Figure 5b, final data point, labeled "l-481-rev*"), far above those produced by *I-Cre*I-expressing fathers.

DISCUSSION

The *rDNA* is composed of the *35S* cistron repeated hundreds of times on each chromosome and is responsible for nucleating the nucleolus, pairing heterogametic sex chromosomes in male meiosis, providing rRNA for ribosome biosynthesis, and modulating protein function through sequestration. Hence the *rDNA* array represents a central regulator in many important aspects of nuclear biology.

TABLE 2

Magnified *Y,rDNA*$^{l\text{-revertant}}$ chromosomes

X chromosome	*Y10B,rDNA*^{l} chromosome	*X/Y* male progeny	*C(1)DX/Y10B*, $rDNA^{l\text{-revertant}}$ female progeny (and phenotypes)
*I-Cre*I	*l-473*	149	3 bb, 3 bb+
*I-Cre*I	*l-480*	196	1 bb, 3 bb+
*I-Cre*I	*l-481*	126	6 (bb)
X	*l-473*	111	—
X	*l-480*	116	1 (bb+)
X	*l-481*	62	1 (bb+)

*I-Cre*I-induced magnifications of the *rDNA* of three *Y* chromosomes previously deleted for the *rDNA*. *I-Cre*I transgene-containing and wild-type (*X*) chromosomes were heat-shocked; only the former expresses *I-Cre*I to create double-strand breaks in the *rDNA*. *X/Y* progeny are normal males, while *C(1)DX/Y10B*, $rDNA^{l\text{-revertant}}$ can survive only if the *rDNA* magnifies. *rDNA* phenotypes refer to the cuticular phenotype of *C(1)DX/Y10B*, $rDNA^{l\text{-revertant}}$ females.

The *rDNA* arrays are regulated such that only about one-half of the cistrons are active, a form of epigenetic regulation thought to involve histone modification; ATP-dependent chromatin remodeling; and, in some organisms, DNA methylation (McStay and Grummt 2008). The proportion of active cistrons can be manipulated by altering gene dosage of important regulators (Mayer *et al.* 2006) or by altering the *in vivo* activity of regulatory enzymes (Sandmeier *et al.* 2002; French *et al.* 2003). Such manipulations have affected cell biology on a large scale because of the centrality of translational capacity, enzymatic modification of chromatin structure, and the nucleolus (Perrin *et al.* 1998). Manipulation of rRNA transcription has been shown to alter gene expression at unlinked sites of the genome, reinforcing the view that the nucleolus is an important determinant in genome regulation (Maillet *et al.* 1996).

What has been absent in these studies is the ability to alter the *rDNA* as easily as the in *trans*-acting regulatory proteins. Molecular genetic analyses of repeated DNA have lagged behind the analysis of single-repeat sequences, in part because of the difficulty of altering repeated sequence *in vivo*. Genetic activities are often redundant, making mutation to recessive phenotypes difficult, and redundant homology does not allow precision during the use of gene targeting. Most past studies of the Drosophila *rDNA* have utilized alleles isolated from unrelated or distantly related sources, which may vary considerably (Lyckegaard and Clark 1989). Even chromosomes isolated from a common stock may differ twofold in *rDNA* content (Averbeck and Eickbush 2005). Although not mapped to the *rDNA*, chromosome polymorphisms can have considerable effects on gene activity (Spofford and Desalle 1991; Lemos *et al.* 2008).

We have developed a method to easily create an isogenic graded allelic series of *rDNA* copy number on the *Y* chromosome of Drosophila, which will circumvent some of these problems. Our approach uses one parental chromosome and derives and characterizes an allelic series within three generations. Further, we have developed a robust assay to quantify the extent of deletion within the *rDNA*. We have shown the efficacy and accuracy of these techniques through genetic and molecular confirmation of damage to the *rDNA*.

The utility of generating deletions within the *rDNA* is manifold, and we have demonstrated one by analyzing natural and induced magnification of the *Y*-linked *rDNA* array in males. Most studies have investigated magnification of the *X*-linked *rDNA* from weakly *bobbed* alleles to wild type in *bobbed* flies, which presumably put some pressure on the *rDNA* to magnify to supply rRNAs. The details of magnification are not understood in Drosophila, although it is clear that some chromosomes are capable of magnification while others are not (Procunier and Tartof 1978; Komma and Endow 1986, 1987). Spontaneous, large increases in *rDNA* array size, in both the germline and the soma, have supported the view that *rDNA* magnification occurs through unequal sister-chromatid exchange (Endow and Komma 1986), although reversion may occur through other means (Marcus *et al.* 1986; Terracol *et al.* 1990). We showed genetically that the reversion of *bobbed* maps to the *Y* chromosome and that the array grows in size concomitant with the reversion.

The qPCR technique that we developed to characterize the array length allowed us to monitor increases in array length in individuals as the *rDNA* underwent magnification. By combining the genetic measurements of translational capacity and the real-time quantitative polymerase chain reaction, we have defined the threshold of *rDNA* copy number necessary for organismal viability. In the absence of an X-linked array, deletions of the *Y*-linked *rDNA* (to ~260 copies) cause a bobbed phenotype or, if more extreme (to ~190), a lethal phenotype. We have established the threshold for the bobbed phenotype by using our allelic series in three different ways. First, deletions defined thresholds of lethal to bobbed and bobbed to wild type. Second, lethal-to-bobbed and bobbed-to-wild-type magnification reinforced those defined limits. Third, magnification by *I-Cre*I expression further reinforced the severe bobbed and mildly bobbed phenotypes. Our findings show that an isogenic chromosome allelic series has clearly defined the limits of phenotypic expressivity, unlike previous studies that revealed broad ranges for these thresholds, further highlighting the utility of our approach.

Arrays allowed to magnify in flies that provide *rDNA* in *trans* exhibit a slow increase in *rDNA* array size that is most consistent with stochastic small increases outnumbering stochastic decreases expected for unequal sister-chromatid exchange between arrays. Thus, the *Y* chromosome *rDNA* arrays are similar to the *X* chromosome arrays: they are capable of magnification, even in the absence of a special inducing chromosome (Ritossa 1968; Hawley and Tartof 1985; Komma and Endow 1986). In previous studies of magnification, not every *Y* chromosome was able to magnify. In fact, a great number of *rDNA* alleles are stable (Lindsley and Zimm 1992). It is possible that a magnifying element similar to the one characterized on the Ybb^0 chromosome existed on both the *Y10A* and *Y10B* chromosomes prior to our work, and deletion merely revealed its presence. It is also possible that induction of *I-Cre*I induced our chromosomes to become magnifying chromosomes, possibly by epigenetic remodeling of the *rDNA* or by activation of resident R1 or R2 transposable elements. Since magnification acts through an unknown mechanism, we cannot state why our chromosomes magnify after reduction.

Hypotheses that *rDNA* quantity is tied to aging, disease, or gene regulation exist but are difficult to test without some way to manipulate the *rDNA* array size (Spofford and Desalle 1991; Gotta *et al.* 1997;

Palumbo *et al.* 1994; Johnson *et al.* 1999; Weber *et al.* 1999; Carmo-Fonseca *et al.* 2000; Martindill *et al.* 2007; Versa-Ostojć *et al.* 2008). Only by altering the initial size of the *rDNA* array, in an otherwise isogenic background, can the contribution of *rDNA* size to these pleiotropic phenomena be investigated. To advance beyond the stage where *rDNA* arrays are merely correlated to aging, cancer, or other diseases or cellular function, variables must be experimentally manipulated. Ideally, this would be done with a minimal perturbation to other factors. Such manipulations—facile, specific, and graded—are now possible with the *rDNA* arrays of Drosophila. If developing hypotheses linking genomewide gene regulation or aging and *rDNA* are correct, then we expect that deletions to the *rDNA* will profoundly affect these phenotypes. Our work has established a mode of generating an allelic series of *rDNA* deletions (or, possibly, expansions) on chromosomes of choice and has detailed a robust means for quantifying the *rDNA* array size. With these chromosomes, it will now be possible to study the role of the *rDNA* in nuclear biology and to address intriguing hypotheses connecting *rDNA* magnification, transcriptional regulation and developmental programs, inheritance of acquired characteristics, and complex diseases or cell states such as cancers or aging.

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