Expression of I-CreI 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 genome-wide 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. Last, we show that I-CreI expression can revert rDNA deletion phenotypes, suggesting double-strand breaks are sufficient to induce rDNA magnification.
INTRODUCTION

The genes that encode three of the four RNA components of ribosomes, the 28S (sometimes called 26S), 18S, 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 approximately one to three hundred 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 et al. 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 fewer than about two hundred (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 cells of an individual (COHEN et al. 2003, COHEN et al. 2005). Wild-type arrays are seen to reduce through so-
matic development, and deleted X-linked arrays to magnify during mitosis and meiosis (DECICCO and GLOVER 1983, HAWLEY and TARTOF 1985), retaining the average of approximately two hundred copies in each array. Measurements of array sizes has 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 using membrane hybridization analyses, which necessitate 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, ROBBINS 1996), most have suffered from an inability to cause specific, graded, and easily-induced damage to the locus. In order 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 the *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-Crel*. We used genetic tests to initially identify deletions to a length below that necessary to serve as 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 define 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 *I-Crel* 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.
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 $I-Crel$-expressing line is $P{v^{h1.8}=hs-I-Crel.R}2A$, $v^1/Y; Sb/TM6b, Ubx$ (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°C and 80% humidity.

Induction and Screen for Deletions. Flies were allowed to lay eggs for 2-3 days, and larvae to develop for one more day. Second and third instar larvae were heat shocked in circulating water baths for at 36°C. In experiments involving Y10A, larvae were heat shocked on two successive days, each treatment lasting 45 minutes. In experiments involving Y10B, larvae were heat shocked on one day for 45 minutes.
Heat shock induced expression was monitored by under-representation of $I$-*CreI* bearing male progeny in relation to $P^{v^{+}t_{1.8}=hs-I-CreI.R}2A$, $v^{1}/y^{1}$ $w^{67c23}$ 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 AGCCTGAGAACGGCTACCA 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, forty cycles alternating between 95°C for 3 seconds and 60°C for 30 seconds. DNA samples were prepared using a modified procedure from K. DOBIE (GLOOR et al. 1993, DOBIE et al. 2003). 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 ten standard deviations above the average of all previous cycles (“no amplification”) fluorescence from each extract, as deter-
mined by the Step-One software. Extracts were run in triplicate (occasionally quadruplicate) identical samples. Samples in discordance with the other samples (a threshold cycle more than two standard errors of the mean different) were interpreted as error 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 CCAACGTGGGGCTCGAAC to generate a 63 nucleotide product. Cycle differences between \(rDNA\) and \(tRNA\) genes ("\(\Delta C_T\)"") 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 \(rDNA^{bb-l}\) 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 are 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, et al. (SULLIVAN et al. 2000).
RESULTS

Creation of rDNA deletions using I-CreI expression

In order 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 EICKBUSCH 2005). We felt 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 $P(v^{1.8}\text{hs-I-CreR})2A$, $v^1/Y10$ 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 I) confirmed that $I$-CreI was expressed, damage occurred to the $X$- and $Y$-linked $rDNA$ arrays, and was subsequently repaired. We reasoned that within the chromosomes we collected by isolating individual male progeny, we would find an allelic series of $rDNA$ deletions.

**A genetic test for $rDNA$ deletion**

Since the $I$-CreI mediated damage to the $Y$ chromosomes is specific to 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, y¹ f¹ bb⁰ (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, rDNA⁰pDf) 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, rDNA⁰pDf chromosomes using this assay, and identified 23 Y chromosomes incapable of supplying sufficient rRNA for survival (1.9%), nine 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 I for summary). Each potential reduction chromosome was retested; those with no or bobbed female progeny again produced like 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 we observed were due to reduction of the \textit{rDNA}, so crossed males from eight of our identified \textit{Y10, rDNA}^\text{pDf} chromosomes (three from \textit{Y10A} and five from \textit{Y10B}) to females of genotype \textit{ln}(1)\textit{sc}^4\textit{sc}^8/\textit{FM7a}, \textit{B}^8. \textit{ln}(1)\textit{sc}^4\textit{sc}^8, like \textit{C}(1)\textit{DX}, lacks \textit{rDNA}. Half of the male progeny of the cross were expected to express a Bar phenotype (\textit{FM7a}/\textit{Y}) and half to be non-Bar (\textit{ln}(1)\textit{sc}^4\textit{sc}^8/\textit{Y10B}, \textit{rDNA}^\text{pDf}), unless the \textit{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 \textit{C}(1)\textit{DX}/\textit{Y10, rDNA}^\text{pDf} lethality or bobbed phenotypes and the \textit{ln}(1)\textit{sc}^4\textit{sc}^8/\textit{Y10B}, \textit{rDNA}^\text{pDf} lethality or bobbed phenotypes (data not shown), indicating that the lethality is linked to the \textit{Y} chromosome and most likely due to \textit{rDNA} deletion, and not the induction of other genomic alterations that interacted with the \textit{C}(1)\textit{DX} background to produce lethality. We confirmed that the only cytologically-visible alteration to the chromosome structures was in \textit{Y}-linked band \textit{h20}, the location of the \textit{rDNA} locus (data not shown).

Since we interpreted our results to mean that all of the identified lines possessed significant reduction of the \textit{rDNA}, these chromosomes will henceforth be referred to as \textit{Y10B, rDNA}^l or \textit{Y10B, rDNA}^bb (or, generically, \textit{Y10B, rDNA}^\text{Df}), consistent with estab-
lished 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. 2005, COHEN et al. 2007), we wished to find a dispersed repeat. For these reasons, we chose the high-copy number *tRNA*\textsuperscript{K-CTT}. Although Lysine is encoded by three anticodons, the CTT isotype is most common, and all fifteen *tRNA* genes of this isotype
(in a haploid genome) have identical sequence (SCHATTNER et al. 2005). Amplification of thirty \( tRNA^{K-CTT} \) 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 approximately 290 (Figure 2, 95% confidence interval 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.

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 the denominator \( tRNA^{K-CTT} \) is able to normalize the signal to 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 fewer than two thousand and a \( tRNA^{K-CTT} \) copy number of approximately 150, however our analyses of \( Y10B, rDNA^{DI} \) presented here are done
with 10-20 ng of total nucleic acid to assure 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 survive to late larvae or early pupae. 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 error 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\textsuperscript{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 former transition occurred at approximately 90% of the hemizygous (Y-linked) rDNA cistron, or approximately 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 is higher than in other studies which investigated the X-linked rDNA bobbed threshold. The X rDNA locus required a deletion to approximately 50 – 80% of the wild-type size, or to about 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 proportion of intact and R1- and R2-
interrupted cistrons (LYCKEAGAARD 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 at approximately 65% of the hemizygous (Y-linked) level of rDNA. This is about 190 copies according to our calculations, and is again higher than previous studies that indicated as few as 114 copies of X-linked rDNA are sufficient for viability (TERRACOL and PRUD’HOMME 1986).

One chromosome, Y10B, rDNA1-539, 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-CreI-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 (TERRACOL and PRUD’HOMME 1981, TERRACOL 1987).

Evidence for rDNA magnification

We initially established stocks of three of the seven rDNA1 deletions derived from Y10A, assuming that they would be stable as stocks containing an X chromosome with
a normal \textit{rDNA} array, since it is generally accepted that magnification of the \textit{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 \textit{X}-linked array. \textit{X}-linked \textit{rDNA} magnification is a well-characterized phenomenon (Marcus et al. 1986), yet we were surprised to see the new \textit{Y10A, rDNA}^l chromosomes exhibited this phenotype after so few generations without obvious selection. Leonard Robbins (Robbins 1981) showed that many deletion alleles of the \textit{rDNA} are stable once generated, while ours are not. Both those alleles and ours rely on creating damage specifically to the \textit{rDNA} arrays, however by different means. Komma and colleagues showed that certain \textit{Y} chromosomes are capable of magnification, even when the cell possesses sufficient \textit{rDNA} (Komma and Endow, 1986, Komma et al., 1993). This feature is not understood but has been shown to reside on some \textit{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 \textit{Y10A, rDNA}^l-3-revertant and \textit{Y10A, rDNA}^l-39-revertant did not map to the \textit{X} chromosome or large autosomes (data not shown). We interpreted these results to indicate that the \textit{Y}-linked \textit{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).

In order to observe magnification as it occurred on our chromosomes, we out-crossed males from each Y chromosome stock to virgins of a common y¹ w⁶⁷c²³ stock to prevent the accumulation of modifiers that could affect rDNA expression. Every generation, we also crossed sibling males to C(1)DX, y¹ f bb⁰/Y or C(1)DX, y¹ f bb⁰/Y, B⁰ females, to genetically assess the status of the rDNA array size in female offspring. This is represented by the “recursive” re-cross in Generation 3 of Figure 1.

We chose to monitor six chromosomes over four generations. Each generation, we extracted DNA and measured rDNA content from 4-10 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 approximately 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\textsuperscript{bb-473} or decreases (e.g., generation two to generation three for Y10B, rDNA\textsuperscript{bb-465}). These results underscore the variation in rDNA magnification amount, but indicate a trend towards progressively larger arrays. Consistent with this, five of the six chromosomes (all but Y10B, rDNA\textsuperscript{bb-473}) showed an increasing coefficient of variance (ratio of standard deviation to average) in each generation. Although some individuals showed arrays which 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 about 15 copies of rDNA per generation, is essentially identical to that observed for the X chromosome (TARTOF 1972).

Magnification is not constant, but may instead increase by small steps, and spend some generations steady in average size. Line Y10B, rDNA\textsuperscript{bb-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 different 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 have either decreased in size, or are not changed appreciably. Over the individuals scored in the sub-
sequent 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 we analyzed, notably Y10B, rDNA^{l-481}, Y10B, rDNA^{l-510}, and Y10B, rDNA^{l-473}, that rDNA size varies between generations, and increase in size is more common than 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 (shown in gray in Figure 4). As predicted, those that were wild-type in appearance had arrays that had increased more than had those that remained bobbed, and were 108% ± 17% of the wild-type quantity of rDNA.

Line Y10B, rDNA^{bb-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-76} females were noticeably less bobbed, and by the following generation, all female progeny had normal cuticles (gray data
points in Figure 4). This corresponds to the generation where the average array size reached 101% ± 8% wild-type level, near the transition we had defined by analyzing the allelic series of initial deficiencies (Figure 3).

We cannot distinguish between somatic “pseudo-magnification,” 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 when a chromosome (e.g., *Y10B*, rDNA^{bb-465} and *Y10B*, rDNA^{bb-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-473} produced two bobbed flies in the second generation, which corresponded to the two Y chromosomes that had very large increases in rDNA size (gray data points). 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 chromatid exchange.
Induction of Magnification by I-Crel 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-Crel.

We crossed three lethal deletions from our allelic series to females carrying an I-Crel transgene and heat shocked for one hour to induce I-Crel 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, rDNA1 chromosomes. If, however, I-Crel expression could induce magnification, we expected to obtain revertant females of genotype C(1)DX/Y10B, rDNA1-revertant. The results are presented in Table 2.

Sires harboring each of the three tested I-Crel-exposed Y10B, rDNA1 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, al-
though some were bobbed+. Since these $Y10B$, $rDNA^l$ were not able to supply sufficient rRNA prior to $I$-Crel 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 $Y10B$, $rDNA^{l-481}$-revertant chromosomes possess a different active-to-inactive ratio of $rDNA$ cistrons than does the original $Y10B$, $rDNA^{l-481}$ chromosome (TERRACOL and PRUD’HOMME 1981, TERRACOL and PRUD’HOMME 1986, TERRACOL 1987, ASHBURNER et al. 2005).

Nondisjunction in the $C(1)DX/\bar{Y}$ 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 $I$-Crel-expressing
sires. Surviving females were bobbed+, indicating that they were such nondisjunctional progeny. Consistent with this, those surviving females had rDNA arrays that were measured to be approximately 200% of the Y10B array (Figure 5b, final data point, labeled “l-481-rev*”), far above those produced by I-Crel-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.

The rDNA arrays are regulated such that only about 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 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 scope 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 has 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 CLARKE 1989). Even chromosomes isolated from a common stock may differ twofold rDNA content (AVERBECK and EICKBUSH 2005). Although not mapped to the rDNA, chromosome polymorphisms can have considerable effects on gene activity (SPOFFORD and DESSALLE 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 \textit{rDNA}. We have shown the efficacy and accuracy of these techniques through genetic and molecular confirmation of damage to the \textit{rDNA}.

The utility of generating deletions within the \textit{rDNA} is manifold, and we have demonstrated one by analyzing natural and induced magnification of the \textit{Y}-linked \textit{rDNA} array in males. Most studies have investigated magnification of the \textit{X}-linked \textit{rDNA} from weakly-\textit{bobbed} alleles to wild-type, in \textit{bobbed} flies which presumably put some pressure on the \textit{rDNA} to magnify in order to supply rRNAs. The details of magnification are not understood in \textit{Drosophila}, although it is clear that some chromosomes are capable of magnification while others are not (PROCUNIER and TAROT 1978, KOMMA and ENDOW 1986, KOMMA and ENDOW 1987). Spontaneous, large increases in \textit{rDNA} array size, in both germline and soma, have supported the view that \textit{rDNA} magnification occurs through unequal sister chromatid exchange (ENDOW and KOMMA 1986), although reversion may occur through other means (MARCUS \textit{et al.} 1986, TERRACOL \textit{et al.} 1990). We showed genetically that the reversion of \textit{bobbed} maps to the \textit{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 \( r\text{DNA} \) underwent magnification. By combining the genetic measurements of translational capacity and the real-time quantitative Polymerase Chain Reaction, we have defined the threshold of \( r\text{DNA} \) copy number necessary for organismal viability. In the absence of an X-linked array, deletions of the Y-linked \( r\text{DNA} \) (to approximately 260 copies) cause a bobbed phenotype or, if more extreme (to approximately 190), a lethal phenotype. We have established the threshold for the bobbed phenotype 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 \( I\text{-CreI} \) expression further reinforced the severe bobbed and mild-bobbed phenotypes. Our findings show that an isogenic chromosome allelic series has clearly defined 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 \( r\text{DNA in trans} \) exhibit a slow increase in \( r\text{DNA} \) 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 (RI-TOSSA 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 Ybb⁰ 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-CreI induced our chromosomes to become magnifying chromosomes, possibly by epigenetic remodeling of the rDNA or 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, PALUMBO et al. 1997, GOTTA et al. 1997, CARMO-FENSECA et al. 2000, MARTINDILL et al. 2000, JOHNSON et al. 1999, WEBER et al. 1999, 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 investi-
gated. In order 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 genome-wide 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, as well as detailed a robust means to quantify the rDNA array size. With these chromosomes, it will now be possible to study the role of the rDNA in nuclear biology and 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.
ACKNOWLEDGEMENTS

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Figure 1.—Genetic Cross and Screen for Y,rDNA Deletions. In Generation 0, females harboring a heat-shock-inducible I-CreI 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-CreI-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 is lacking 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 bobbed female phenotypes (in Generation 3). 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-CreI is P{v^{+1.8}=hs-I-CreI.R}2A, v¹; y w is y¹ w^{67c23}; Y¹0 is either y⁺yw⁺, Dp(1;Y) y⁺, P{w^=RSw}¹0A or y⁺yw⁺, Dp(1;Y) y⁺, P{w^=RSw-}¹0B; C(1)DX is C(1)DX, y¹ f¹ bb⁰.
Figure 2.—Real-time “quantitative” polymerase chain reaction (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 average ± standard error of the mean ranges for amplification cycles (Ct). Rightmost graph shows 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 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 2ΔΔCt. Y axes are either cycles of qPCR or differences in cycles between different samples.
Figure 3.—Y, rDNA<sup>Dr</sup> 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 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. To the left of the graph is indicated the approximate range that corresponds to those phenotypes. 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 bobbed phenotype.

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 (Xs), as well as the average of the population (+s, connected by lines). Black data indicate individuals with the same phenotype (lethal or bobbed) as previous generations, gray data are from individuals whose phenotype changed (to wild-type for bb-
465, or to bobbed for l-473). X axes are successive generations after establishment as stock. Y axes are ratio to wild-type Y10B chromosome rDNA content. For clarity, standard errors are not depicted.

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

Paredes and Maggert
Figure 2
Paredes and Maggert
Figure 3

Paredes and Maggert
Figure 4
Paredes and Maggert
Figure 5

Paredes and Maggert
Table 1. Number of Derived $Y,rDNA^{Df}$ Chromosomes.

<table>
<thead>
<tr>
<th>Screen</th>
<th>Parental Y Chromosome</th>
<th>Chromosomes Screened</th>
<th>Altered Sex Ratio</th>
<th>$T(X;Y)$</th>
<th>$Y,rDNA^{bb}$</th>
<th>$Y,rDNA^{l}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y10A</td>
<td>560</td>
<td>32</td>
<td>12</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Y10B</td>
<td>600</td>
<td>60</td>
<td>6</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td>1160</td>
<td>92</td>
<td>18</td>
<td>9</td>
<td>23</td>
</tr>
</tbody>
</table>

*l-Crel*-induced deletions within the $rDNA$ of two related $Y$ chromosomes. Y10A and Y10B differ only by a $white^c$ 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.
Table 2. Number of Magnified $Y_{rDNA}$-$\text{rDNA}^{\text{I-revertant}}$ Chromosomes.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>$Y10B_{rDNA}$ Chromosome</th>
<th>$X/Y$ male progeny</th>
<th>$C(1)DX/Y10B$, $rDNA_{rDNA}^{\text{I-revertant}}$ female progeny (and phenotypes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$l$-Crel</td>
<td>$l$-473</td>
<td>149</td>
<td>3 bb, 3 bb+</td>
</tr>
<tr>
<td>$l$-Crel</td>
<td>$l$-480</td>
<td>196</td>
<td>1 bb, 3 bb+</td>
</tr>
<tr>
<td>$l$-Crel</td>
<td>$l$-481</td>
<td>126</td>
<td>6 (bb)</td>
</tr>
<tr>
<td>$X$</td>
<td>$l$-473</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X$</td>
<td>$l$-480</td>
<td>116</td>
<td>1 (bb+)</td>
</tr>
<tr>
<td>$X$</td>
<td>$l$-481</td>
<td>62</td>
<td>1 (bb+)</td>
</tr>
</tbody>
</table>

$l$-Crel-induced magnifications of the $rDNA$ of three $Y$ chromosomes previously deleted for the $rDNA$.

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