Clustering of rDNA containing type 1 insertion sequence in the distal nucleolus organiser of *Drosophila melanogaster*: implications for the evolution of X and Y rDNA arrays

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Summary

The ribosomal RNAs produced by the multigene families on the X and Y chromosomes of *Drosophila melanogaster* are very similar despite the apparent evolutionary isolation of the X and Y chromosomal rDNA. X–Y exchange through the rDNA is one mechanism that may promote co-evolution of the two gene clusters by transferring Y rDNA copies to the X chromosome. This hypothesis predicts that the proximal rDNA of X chromosomes will be Y-like. Consequently, rDNA variants found only on the X chromosome (such as those interrupted by type 1 insertions) should be significantly clustered in the distal X nucleolus organizer. Proximal and distal portions of the X chromosome nucleolus organizer were separated by recombination between the inverted chromosomes $In(1)sc^{V2}$ (breakpoint in the centre of the rDNA) and $In(1)sc^{4L}sc^{8R}$ (no rDNA). Molecular analyses of the resulting stocks demonstrated that rRNA genes containing type 1 insertions were predominantly located on the chromosome carrying the distal portion of the X rDNA, thus confirming a prediction of the X–Y exchange hypothesis for the co-evolution of X and Y chromosomal rDNA. Distal clustering is not predicted by the alternative hypotheses of selection or gene conversion.

1. Introduction

The ribosomal RNA genes (rDNA) in *Drosophila melanogaster* occur in tandem arrays of about 250 copies at the nucleolus-organizing regions on both the X and Y chromosomes (Tartof, 1975; Ritossa, 1976). About half the rRNA genes on the X chromosome are interrupted by type 1 (T1) insertions, but no T1 insertions are found on the Y chromosome (Tartof & Dawid, 1976). T1 insertions also occur in tandemly repeated arrays outside the nucleolus organizer primarily in the distal X heterochromatin (Dawid & Botchan, 1977; Kidd & Glover, 1980; Peacock *et al.*, 1981; Appels & Hilliker, 1982). A second class of insertions (type 2) interrupt about 15% or rDNA repeats on both the X and Y chromosomes (Wellauer, Dawid & Tartof, 1978; Roiha & Glover, 1980: Long, Rebbert & Dawid, 1980).

In the absence of meiotic crossing over in male *D. melanogaster*, the X and Y nucleolus-organizing regions should be evolutionarily independent and diverge. Despite this expectation, the rRNA coding sequences on the X and Y chromosomes are very similar, if not identical (Maden & Tartof, 1974;

Yagura, Yagura & Maramatsu, 1979). Further, the X and Y chromosomes in long-established stocks share rDNA spacer classes (Wellauer *et al.* 1978; Coen, Thoday & Dover, 1982; Boncinelli *et al.* 1983), internal transcribed spacers (Coen, Strachen & Dover, 1982) and classes of type 2 insertions (Tartof & Dawid, 1976; Wellauer *et al.* 1978).

How can the similarities and differences of the X and Y chromosomal rDNA be explained? Three hypotheses have been proposed to account for the co-evolution of X and Y chromosomal rDNA. Tartof & Dawid (1976) proposed that similar selection pressures caused the co-evolution of X and Y nucleolar-organizer regions. While rRNA coding sequences are strongly constrained by selection (see Gerbi, 1985; Pace, Olsen & Woese, 1986), it seems unlikely that every one of the thousands of bases have critical functions such that all are constrained by selection. Certainly the similarities in rDNA spacers, internal transcribed spacers and type 2 insertion sequences cannot be explained by selection alone.

Gene conversion has also been proposed as a mechanism for the co-evolution in multigene families (see Baltimore, 1981). Gene conversion would be expected to cause bi-directional transfer of variants between the rDNA on the X and Y chromosomes. T1 insertions should enter the Y rDNA as readily as Y-

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like variants enter the X rDNA. As T1 insertions are not found on the Y chromosome, gene conversion appears insufficient to account for the co-evolution of X and Y rDNA. However, it is possible that lack of homology may prevent type 1 insertion sequences from being transferred by gene conversion.

The third hypothesis for the co-evolution of X and Y rDNA is the $X–Y$ exchange hypothesis (Wellauer *et al.* 1978; Maddern, 1981). Exchanges (translocations) between X and Y chromosomes at the nucleolus-organizing regions will generate compound chromosomes with X and Y rDNA. When exchange occurs in the orientation illustrated in Fig. 1, two compound chromosomes are generated: a $Y^S X$. which functions neither as an X nor a Y, and an $X.Y^L$ which behaves like an X chromosome. These $X–Y$ exchange products have been detected by Coen & Dover (1983) and Gillings *et al.* (1987) in *Drosophila* lines selected for low abdominal bristle number (Frankham, Briscoe Nurthen, 1978, 1980; Frankham, 1980). The $X–Y$ exchange results in Y chromosomal rDNA being donated to the X chromosome but not vice versa. This Y chromosomal rDNA can enter cytologically 'normal' X chromosomes by the $X.Y^L$ chromosome losing the Y^L arm, as observed by Gillings *et al.* (1987). Alternatively, exchange between $X.Y^L$ and normal X chromosomes may result in such transfer.

The $X–Y$ exchange hypothesis predicts that rDNA in the proximal portion of the X nucleolus organizer will be Y-like, and that rDNA in the distal portion of the X nucleolus organizer will be X-like. Consequently, this hypothesis predicts that T1 insertions will be clustered distally in the nucleolus organizers of normal X chromosomes. Evidence for the clustering of variants within the X nucleolus organizer is equivocal. Renkawitz-Pohl, Glatzer & Kunz (1981), Sharp, Gandhi & Procunier (1983), Kalumuck & Procunier (1984), Salzano & Malva (1984), Terracol & Prud'homme (1986) and Gillings *et al.* (1987) have reported evidence for clustering of rDNA containing T1 insertions in *D. melanogaster* and *D. hydei*, but they did not determine the location of the clusters. Conversely, De Cicco & Glover (1983), Hawley & Tartof (1983), Palumbo, Endow & Hawley (1984) and Dutton & Krider (1984) have all claimed that rRNA genes containing T1 insertions are randomly dispersed throughout the X chromosomal nucleolus organizer. Gillings *et al.* (1987) pointed out shortcomings in interpretations in the latter studies. Hilliker & Appels (1982) reported that about 90% of T1 insertions were clustered in the distal nucleolus organizer and heterochromatin of the *In(1)sc^{V2}* chromosome. However, they could not distinguish between T1 insertions located in heterochromatin and those inserted into the rDNA.

The objective of this work was to determine whether rDNA containing T1 insertions is predominantly in the distal nucleolus organizing region of the X chromosome of *D. melanogaster* as predicted by the
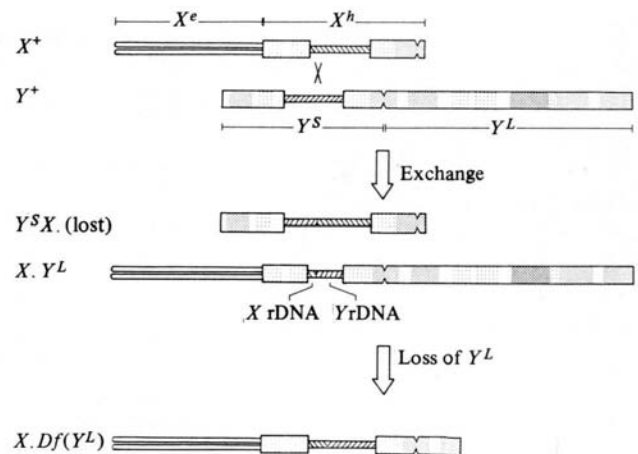

Fig. 1. Consequences of $X–Y$ exchange through the nucleolus-organizing regions of the X and Y chromosomes. $X^e = X$ chromosome euchromatin, $X^h = X$ heterochromatin. All the Y chromosome is heterochromatic. The cross-hatched regions are the nucleolus-organizing regions (rDNA), the narrow constrictions the centromeres and the stippling represents the Hoechst banding patterns.

$X–Y$ exchange hypothesis for the co-evolution of X and Y rDNA. To investigate this, distal and proximal portions of the nucleolus organizer were separated using an X chromosomal inversion with a breakpoint in the centre of the nucleolus organizing region. rRNA genes containing T1 insertions are located predominantly in the distal portion of the X nucleolus organizer.

2. Materials and methods

(i) *Stocks*

The following chromosomes were used:

In(1)sc^{V2}. This inversion has one breakpoint in the centre of the nucleolus-organizing region and another near the scute locus so that the distal rDNA is located near the tip of the chromosome and the proximal rDNA near the centromere (Lindsley, Appels & Hilliker, 1982).

In(1)sc^{4L}sc^{8R}, y Tu w (XNO^-). This chromosome has no rDNA.

In(1)sc^{V2L}sc^{8R}, y Tu (XNO^p). This chromosome has only the proximal portion of the nucleolus-organizing region.

In(1)sc^{4L}sc^{V2R}, w (XNO^d). This chromosome has only the distal portion of the nucleolus-organizing region.

C(1)DX, yf. This attached-X chromosome has no rDNA.

B^S Y.

In(1)dl-49, v Bx^3 mal.

The above chromosomes and mutations are described by Lindsley & Grell (1968), with the exception of the third and fourth stocks whose construction is described below.

(ii) Construction of XNO^p and XNO^d stocks

To separate the proximal and distal portions of the X-chromosome nucleolus-organizing region, the inversions $In(1)sc^{V2}$ and $In(1)sc^{4L}sc^{8R}$, $y\,Tu\,w$ were crossed and the appropriate recombinants recovered with the aid of the markers y, Tu and w (Fig. 2). $In(1)sc^{V2L}sc^{8R}$, w (XNO^d) contains the distal portion of the nucleolus organizer and adjacent heterochromatin, and $In(1)sc^{4L}sc^{V2R}$, $y\,Tu$ (XNO^p) contains the proximal portion of nucleolus organizer and adjacent heterochromatin.

Stocks of each recombinant X chromosome were constructed by mating single recombinant males to $C(1)DX$, yf/B^sY females.

(iii) Generation of genotypes for molecular analysis

To avoid having to account for the rDNA contributed by chromosomes other than those of interest, the initial experiments were carried out using homozygous females of $In(1)sc^{V2L}sc^{8R}$ and $In(1)sc^{4L}sc^{V2R}$, and these same chromosomes heterozygous with the rDNA deletion chromosome, $In(1)sc^{4L}sc^{8R}$. Attempts to utilize XO males were unsuccessful due to the very low fitness of $In(1)sc^{V2L}sc^{8R}/O$ males, probably as a result of extreme position-effect variegation for the *achaete*
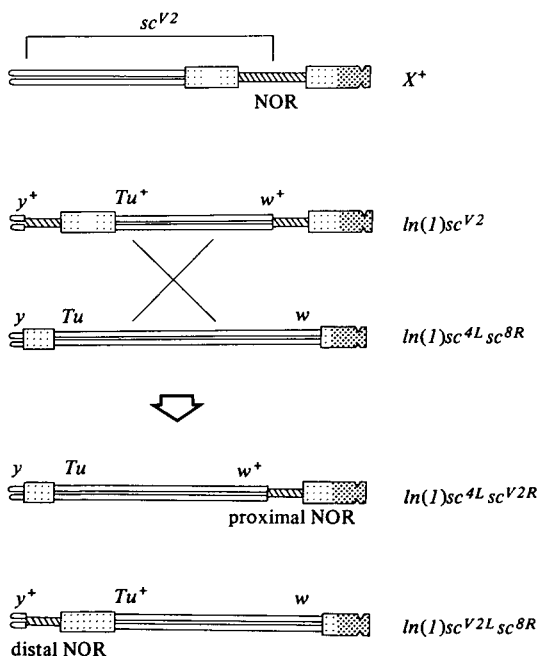

Fig. 2. Procedure used to separate the distal and proximal portions of the nucleolus-organizing region (rDNA) of the $In(1)sc^{V2}$ chromosome. The upper X^+ chromosome represents the normal X chromosome, with the bracketed region showing the breakpoints of the $In(1)sc^{V2}$ inversion. The $In(1)sc^{V2}$ inversion was recombined with marked $In(1)sc^{4L}sc^{8R}$ chromosomes (lacking rDNA) and recombinant chromosomes containing either the proximal or the distal portions of nucleolus-organizing region (NOR) collected. Chromosome landmarks are described in Fig. 1.
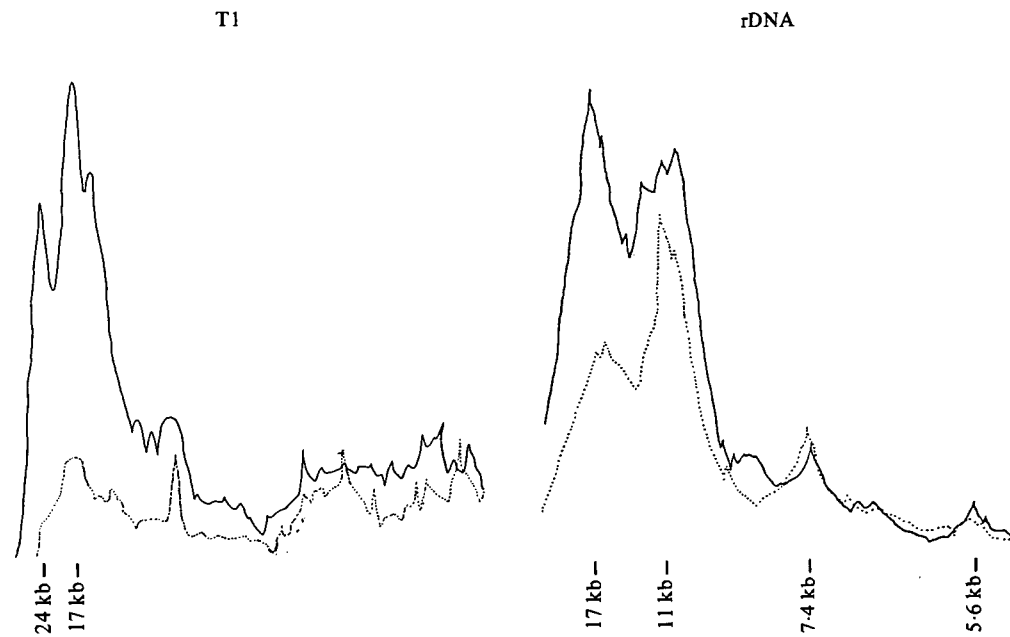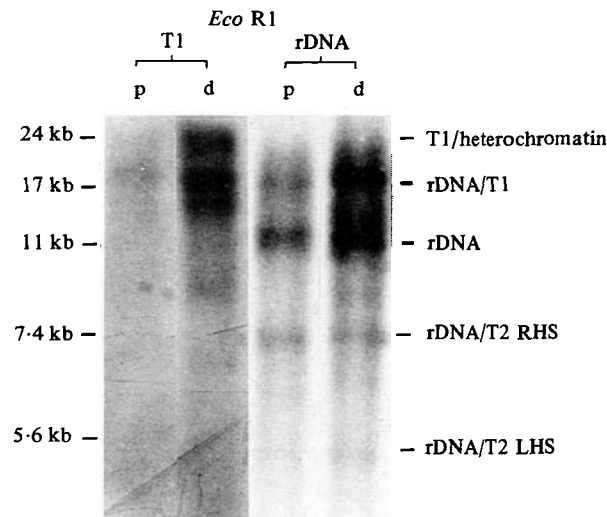
locus. The generation of homozygous and deletion heterozygote genotypes is described below:

(1) XNO^p/XNO^- and XNO^d/XNO^- females were generated by mating $In(1)dl-49, v\,Bx^3\,mal/In(1)sc^{4L}sc^{8R}$, $y\,Tu\,w$ females to XNO^p/B^sY and XNO^d/B^sY males and collecting the required F1 genotypes.

(2) Homozygous XNO^p and XNO^d stocks were produced by mating $In(1)dl-49, v\,Bx^3\,mal/In(1)sc^{4L}sc^{8R}$ $y\,Tu\,w$ females to either XNO^p/B^sY or XNO^d/B^sY males, and taking $In(1)dl-49/XNO^p$ or $In(1)dl-49/XNO^d$ F1 females and backcrossing them to XNO^p/B^sY or XNO^d/B^sY males, respectively. Homozygous females and hemizygous males were used to establish homozygous stocks.

In a later experiment to determine whether independent recombinants yielded similar results, XNO^p/B^sY, XNO^d/B^sY and $In(1)sc^{4L}sc^{8R}/B^SY$ males were subjected to molecular analyses.

(iv) Molecular analyses

DNA was extracted from flies of each genotype, digested with the restriction enzyme *Eco* R1, separated on an agarose gel and transferred to a nylon membrane (Zeta Probe, Biorad). Transfers were probed with P^{32}-labelled rDNA (*pDm238*; Roiha *et al.* 1981) or T1 (*pC225*; Roiha & Glover, 1980) probes. Loadings of DNA in the different tracks were equalised. Samples from the different DNA extracts were run on minigels and stained with ethidium bromide. Sample volumes used for loading the gels described herein were adjusted correspondingly to achieve equal concentrations of DNA. Details of all the methods can be found in Gillings *et al.* (1987).

Eco RI and *Hin*d III digests of λ DNA were used as marker tracks. A small amount of λ DNA was added to the nick translation reaction to detect the λ bands in the subsequent autoradiograph. λ DNA shares no homology with *Drosophila* DNA (data not shown).

(v) Densitometry

Tracks were scanned using a LKB2202 Laser Densitometer.

3. Results and discussion

The *Eco* R1 rDNA restriction patterns of homozygous stocks containing either the proximal (XNO^p) or distal (XNO^d) rDNA of $In(1)sc^{V2}$ are shown in Fig. 3. Filters were probed with rDNA and T1 probes to distinguish the different rDNA repeat types.

Bands hybridizing only to the T1 probe originate from tandem T1 repeats in the heterochromatin. Such tandem repeats are bounded by, but do not contain *Eco* R1 sites, thereby generating large fragments [about 24 kilobases (kb), Fig. 3].

Three major fragments hybridize only to the rDNA probe. The 11.5 kb fragment corresponds to the

Fig. 3. *Eco* R1 digests of genomic DNA from homozygous XNO^p (p) and XNO^d (d) stocks probed with T1 or rDNA probes. Autoradiographs are shown above the densitometer scans below. Solid lines, XNO^d; dotted lines, XNO^p

uninterrupted rRNA genes, while the 7.4 and 5.6 kb fragments represent the two halves of rRNA genes containing T2 insertions (see Gillings *et al.* 1987). The major fragment hybridizing to both the rDNA and T1 probes is 17 kb in length. This corresponds to the rRNA genes containing T1 insertions. In addition, there may be other less frequent rRNA genes containing T1 insertions at 13–14, 9 and 6 kb.

The significant finding is that rRNA genes interrupted by T1 insertions are primarily in the distal portion of the nucleolus-organizing region of $In(1)sc^{V2}$. The higher levels of the rDNA containing T1 insertions in XNO^d than XNO^p is obvious for the 17 kb fragment. Further, the 13–14 kb band and the 9 kb band, both seem to be unique to XNO^d. Simi-

lar results were obtained for deletion heterozygotes (results not shown).

In contrast to the clustering of rRNA genes containing T1 insertions, rRNA genes containing T2 insertions are at similar levels in the distal and proximal portions of the nucleolus organizer.

A comparison of results for five independent recombinants of the XNO^p and XNO^d classes (Fig. 4) demonstrate that the distal clustering is a consistent feature for all recombinants. Consequently, polymorphism within the $In(1)sc^{V2}$ stock can be dismissed as a cause of the evidence for distal clustering of rRNA genes containing T1 insertions presented above.

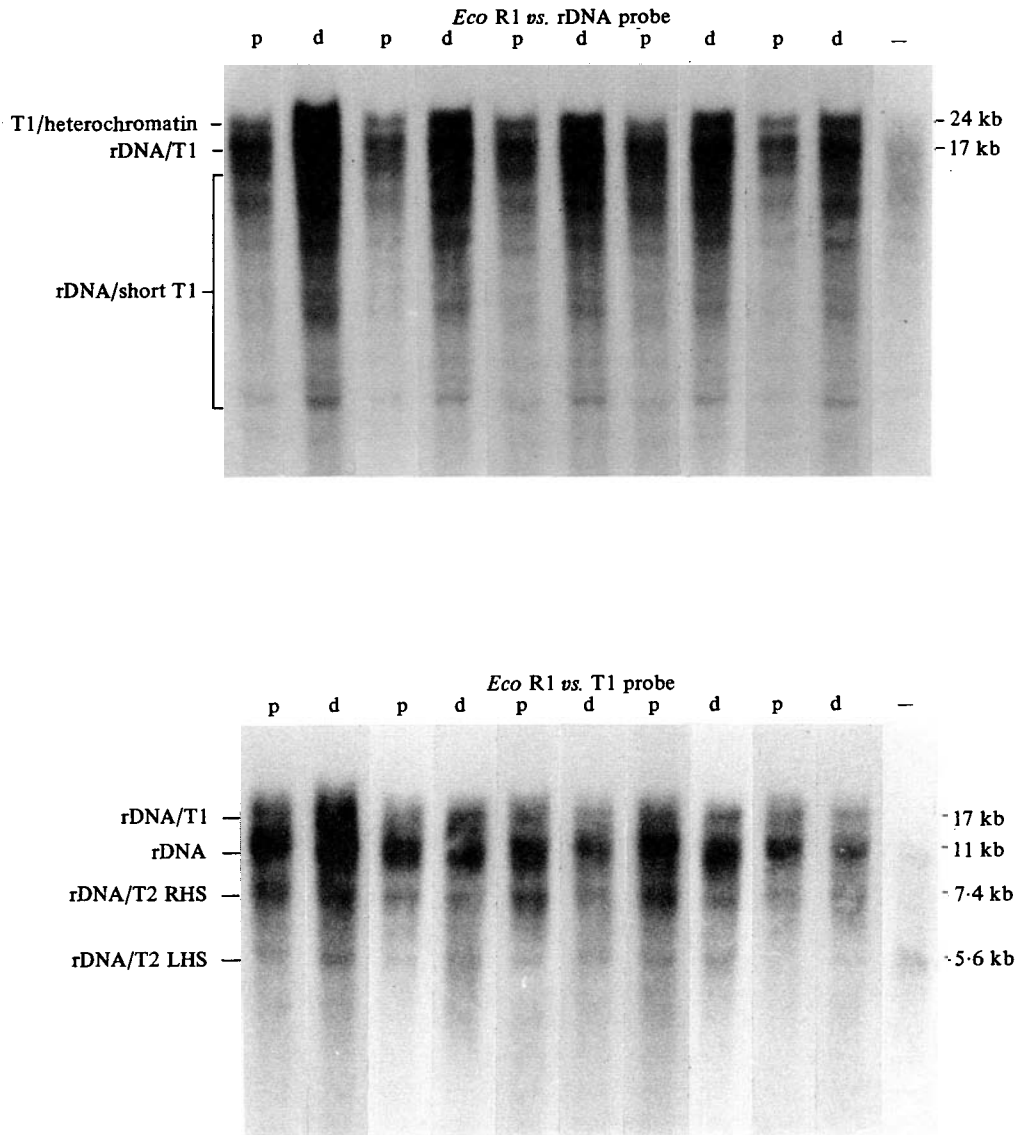Quantitation of the levels of rDNA repeats con-

Fig. 4. *Eco* R1 digests of genomic DNA from XNO^p/B^sY (p) and XNO^d/B^sY (d) males of five independent recombinants of each type plus XNO^-/B^sY. —, Probed with rDNA and T1 probes.

taining or not containing T1 insertions in the two portions of the nucleolus-organizing region was done using densitometer scans. Densitometer scans for homozygotes probed with rDNA or T1 probes are shown in the bottom portion of Fig. 3. Similar scans were done for the autoradiographs presented in Fig. 4 (not shown). As loadings of DNA were equalised, the rDNA probing shown in Fig. 4 indicates that the total amounts of rDNA in XNO^p and XNO^d were not distinguishably different, agreeing with the assertion that the breakpoint is almost is the middle of the rDNA (Lindsley *et al.* 1982). The level of rDNA containing T1 insertions was estimated by subtracting the heterochromatic T1 class from the total response due to T1 insertions and dividing by the rDNA response for the same track (to adjust for minor differences in DNA loading). The resulting figures for the homozygotes indicate that XNO^d had 72% of the rRNA genes contained T1 insertions and XNO^p 28%,

and the corresponding figures obtained from the tracks containing X/B^sY males were the same. Consequently, about 2·6 times as many rRNA genes containing T1 insertions are to be found in the distal rDNA as compared to the proximal rDNA.

These findings corroborate evidence that most of the rRNA genes interrupted by T1 insertions are clustered in one or a few tandem arrays (see Introduction) and locate these predominantly in the distal portion of the nucleolus organizing region as suggested by the results of Hilliker & Appels (1982). It should be noted that our results preclude the location of all such genes on the distal boundary of the nucleolus-organizing region as there are rRNA genes without insertions in the distal portion of the nucleolus-organizing region and rRNA genes with T1 insertions in the proximal region.

Can the $In(1)sc^{V2}$ chromosome have undergone any evolution in the time since its induction in 1946 (see

Lindsley & Grell, 1968) that could effect the validity of the assumptions made here? The $In(1)sc^{V2}$ chromosomes carried by the stocks used in this experiment are descended from a single γ-ray induced inversion chromosome that has passed through 500–1000 generations since then. Homogenization of proximal and distal rDNA by recombination should not occur because the proximal and distal halves of the nucleolus-organizing region are separated at opposite ends of the $In(1)sc^{V2}$ chromosome. The two halves are therefore expected to diverge. The proximal rDNA in this stock should have been able to continue to exchange with Y chromosomal rDNA by X–Y exchange, but the distal rDNA in this chromosome should be isolated from further X–Y exchange events. This does not in any way negate the interpretations presented here. It implies that the differences observed may not have been the same ones that existed when the inversion was induced. There is one way in which the results could be an artifact of evolution in the inversion chromosome. If transposition of T1 sequences into rDNA requires physical proximity, then the distal rDNA may have accumulated additional T1 insertions during its evolution as it is adjacent to the heterochromatic T1's, while the proximal rDNA is distant from them near the other end of the chromosome. However, there is currently no evidence that physical proximity is required for transposition of T1 sequences and only circumstantial evidence that they transpose at all (see Gillings *et al.* 1987).

Do these results apply to chromosomes other than $In(1)sc^{V2}$? Molecular evidence for clustering of rRNA genes containing T1 insertions exists for several chromosomes as discussed above. While this evidence does not prove that distal clustering is to be found in other chromosomes, it can be considered circumstantial evidence that it does.

The results presented confirm the prediction of the X–Y exchange hypothesis for the co-evolution of X and Y rDNA that rDNA proximal to the centromere will be Y-like and the rDNA distal to the centromere will be X-like. Distal clustering is not predicted by the alternative hypotheses of selection and gene conversion.

These findings reinforce other evidence that support the X–Y exchange hypothesis, namely:

(1) X–Y exchange through the rDNA has been shown to occur (Coen & Dover, 1983; Gillings *et al.* 1987).

(2) An $X.Y^L$ chromosome, the product of an X–Y exchange, has been found in a wild population (Gillings *et al.* 1987).

(3) The $X.Y^L$ chromosome must lose the Y arm or be at a selective disadvantage to normal X chromosomes to retain the normal morphology of the X chromosome. Gillings *et al.* (1987) presented evidence for the loss of part or all of the Y^L arm from $X.Y^L$ chromosomes. Further, Frankham (unpublished) has evidence that $X.Y^L$ chromosomes are at a selective

disadvantage in competition with the same X chromosome without the Y^L arm.

The evolution of rDNA in *Drosophila* is most probably affected by selection, genetic drift, equal and unequal X–X exchanges, X–Y exchanges, and gene conversion and by transposition of insertion sequences. The above mentioned evidence indicates that X–Y exchange plays a major role in the co-evolution of X and Y chromosomal rDNA.

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