

Distribution of LINEs and other repetitive elements in the karyotype of the bat *Carollia*: implications for X-chromosome inactivation

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This article is dedicated to Professor Karl Fredga to commemorate his retirement and lifelong cytogenetic research activities.

Abstract. The Lyon repeat hypothesis postulates that long interspersed elements (LINEs) play a role in X-chromosome inactivation. Evidence to support this hypothesis includes the observation that the degree of inactivation of autosomes translocated to the X chromosome is correlated with LINE density on that autosome. We examined the distribution of LINEs in the fruit bat *Carollia brevicauda*, which has an autosomal translocation to the X that occurred at least 7 million years ago. A quantitative analysis of LINE accumulation on multiple metaphase chromosome spreads revealed a significant accumulation on the original X relative to the attached autosome, the

homolog of that autosome (Y_2), and chromosome 1. Previous replication studies indicate that for the X and attached autosome, only the original X chromosome replicates late in *Carollia* females, and that the attached autosome replicates in the same timeframe as other autosomes. These data are compatible with the Lyon repeat hypothesis, and the possibility that LINEs act as booster elements for X inactivation remains a viable hypothesis. We address the procedures and limitations of quantitative analysis based on in situ hybridization.

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Compared to the autosomes, the X chromosome has a lower density of genes, a lower mutation rate, and higher copy number of some repetitive elements—especially the long interspersed element, LINE-1. Furthermore, dosage compensation in mammals is achieved by inactivation of one X chromosome in females, resulting in differences in gene expression between the two X chromosomes, and is associated with late replication, methylation, and hypoacetylation of the inactive X. The observation that LINEs are enriched on the X chromosome has led to the hypothesis that these elements may play a role in X inactivation and dosage compensation (Lyon, 1998a, b). Mechanisms proposed to explain the accumulation of repetitive sequences on the X chromosome include differences between the autosomes and the X in the rate of crossingover, and thus the rate of removal of elements by ectopic excision (Langley et al., 1989; Baker and Wichman, 1990), preferential insertion into late-replicating regions of the genome, or sequence-specific insertion (Wichman et al., 1992).

X-chromosome inactivation is initiated at a region of the X chromosome (XIC, or X inactivation center) and involves transcription of a large RNA from the XIST (X inactivation-specific transcript) locus (Brown, 1991, 1992). Unlike other X-linked genes, the XIST gene product is transcribed only from the inactivated X and appears to coat the inactivated X chromosome. Several lines of evidence suggest that there is something fundamentally different about the X chromosome (versus the autosomes) with respect to XIST spreading. First, transgenic introduction of the XIC into an autosome results in some degree of inactivation of that autosome, but the inactivation

homolog of that autosome (Y_2), and chromosome 1. Previous replication studies indicate that for the X and attached autosome, only the original X chromosome replicates late in *Carollia* females, and that the attached autosome replicates in the same timeframe as other autosomes. These data are compatible with the Lyon repeat hypothesis, and the possibility that LINEs act as booster elements for X inactivation remains a viable hypothesis. We address the procedures and limitations of quantitative analysis based on in situ hybridization.

Supported in part by a grant from Mr. James Sowell and by a grant from National Institutes of Health grant GM38737 to H.A.W. P.V. was supported by a grant from the National Science Foundation (EPSCoR) to James Foster.

Received 19 February 2002; revision accepted 8 April 2002.

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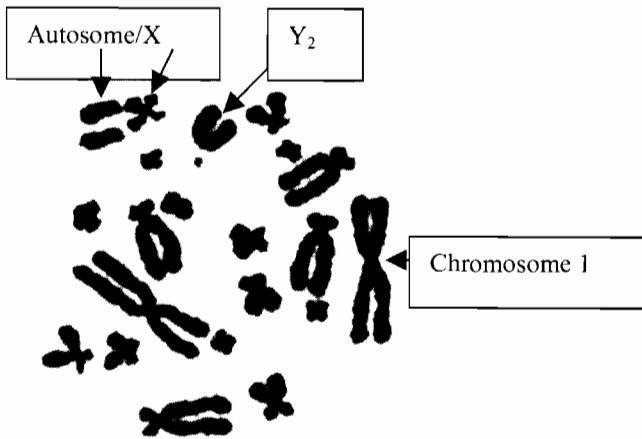


Fig. 1. Karyotype of a *Carollia* showing the size and centromere placements. The four chromosomes that are quantified for LINE signal are: (1) the largest autosome (chromosome 1); (2) the original X (X_0); (3) an autosome that is translocated to the distal end of the X_0 (X_A); and (4) Y_2 , which is the autosome homologous to the translocated segment. The Y_1 , which is the original Y, is the small chromosome immediately below Y_2 .

appears to be less complete and less stable than X inactivation and requires multiple copies of the transgene (Heard, 1996, 1999a, b). Second, translocation of autosomal segments onto the X does not always result in inactivation of those translocated segments (Duthie, 1999). In human cell lines carrying an autosome-X translocation, the late replication of the inactivated X sometimes spreads into the translocated autosomal segment, but is less complete than it is for the X chromosome (White, 1998).

Gartler and Riggs (1983) hypothesized that transmission of the inactivation signal along the X involves some sort of booster elements that facilitate spreading of XIST. The observation that inactivation can, under some circumstances, spread into a translocated or transgenic autosome suggests that these booster elements may not be exclusive to the X, but rather may be enriched there. Lyon (1998a, b) hypothesized that LINE-1s may be the booster elements. Several lines of evidence support this hypothesis: (1) In situ hybridization in humans (Korenberg and Rykowski, 1988) and rodents (Baker and Wichman, 1990; Boyle, 1990) suggests that LINEs are enriched on the X chromosome relative to the autosomes. (2) In humans, enrichment on the X has been confirmed by computer-aided analysis of portions of the X and several autosomes (Bailey, 2000). Enrichment on the X is greatest for the younger LINE inserts. (3) There is a relative deficiency of LINEs in the region of human genes that escape X inactivation (Bailey, 2000). About 15% of the genes on the human X escape inactivation (Carrel, 1999), whereas the number that escape inactivation in the mouse appears to be much smaller (Disteche, 1995, 1999; Tsuchiya, 2000). The degree of X inactivation correlates with the level of recent LINE transposition in each species, which has been higher in mouse than in human (Furano, 2000). (4) Other genomic regions that are extremely rich in LINEs show some characteristics of heterochromatin. For example, a region of high LINE density is observed on the short arm of

chromosome 1 in the deer mouse, *Peromyscus*, and does not correspond to a G-band (Baker and Kass, 1994). This region appears to be intermediate in staining between euchromatin and heterochromatin and was originally described as heterochromatic (Arrighi et al., 1976). (5) Although the data are somewhat limited, there is a correlation between the spreading of X inactivation into translocated autosomes and the density of LINEs on these autosomes (Lyon, 1998a, b).

There are examples of translocations to the X during evolution. If LINE-1s play a role in X inactivation, then only autosomes with a low LINE-1 density should be tolerated when translocated to the X chromosome during evolution. These translocated segments should maintain a lower density of LINE-1s than the X and should not undergo inactivation with the true X.

In the fruit bat *Carollia*, there is a large autosome translocated onto the X (Hsu et al., 1968). *C. brevicauda* was chosen as a model for several reasons. First, the diploid number is low ($2n = 20-21$), and, furthermore, considerable size and centromere placement variation permits discrimination among chromosomes (Fig. 1; Baker, 1967; Hsu et al., 1968). One submetacentric autosomal pair is much larger than any other chromosomes in the karyotype. Second, an acrocentric autosome that is larger than the original X has been translocated to the X. Between the original X and the translocated autosome is a site of ribosomal genes, which permits clear delineation between the X and the autosome. Additionally, in males the homolog of the autosome translocated to the X is the only medium-sized acrocentric in the karyotype. This acrocentric has not been translocated to the Y, resulting in an XX/XY_1Y_2 sex-chromosome system (Baker, 1967; Hsu et al., 1968; Fredga, 1970). The aforementioned combination of chromosomal features (Fig. 1) permits positive identification of a large autosome (1), the original X (X_0), the autosomal region translocated to the X (X_A), and the autosomal homolog of the X translocation, or neoY (Y_2 , following the nomenclature of Fredga, 1970).

We incorporated two probes in our study on the accumulation of repetitive elements: a LINE-1 probe and a *C. brevicauda* total genomic probe. The LINE probe is used to visualize the copy number of LINEs in the bat genome. The *Carollia* total genomic DNA probe is employed to visualize regions of the karyotype with low levels of LINEs but high levels of other repetitive elements.

Materials and methods

Karyotype preparation

Karyotypes were prepared from two *C. brevicauda* individuals (TK104312, TK104313) caught using mist nets in natural populations. Individuals were injected with vinblastine sulfate (Velban) and sacrificed 1½ h later, and bone marrow was isolated from the humerus. Karyotypes were prepared the morning after capture using methods described by Baker and Qumsiyeh (1988), except that the time in hypotonic solution was decreased to 16 min. After a final wash in Carnoy's fixative, cell suspensions were maintained in 2-ml Eppendorf tubes at room temperature. Once returned to the laboratory at Texas Tech University, cell suspensions were stored at -20°F until slides were made by the blaze-dry method for in situ hybridization. A Giemsa-stained *Carollia* karyotypic preparation is shown in Fig. 1. The autosome translocated to the original X is clearly demarcated by a secondary constriction.

Origin of probes and in situ hybridization

The LINE probe Cper2 (535 bp) was isolated from *C. perspicillata* genomic DNA (TK104347, GenBank accession number AY089964), using the method described by Cantrell et al. (2000). *C. brevicauda* and *C. perspicillata* (for the population from which the probe was isolated) are sister taxa with a genetic distance value of 4.2% for the cytochrome b gene (Wright et al., 1999). The genomic DNA was isolated from liver tissue of *C. brevicauda* (TK104530). Probes for in situ hybridization were labeled by standard nick translation with biotinylated dATP using a BioNick nick-translation kit and following the manufacturer's (GIBCO BRL) instructions. Hybridization followed the procedures as previously described (Moyzis et al., 1987, 1988; Baker and Wichman, 1990) using fluorescein avidin and biotinylated anti-avidin. Hybridization patterns were examined using an Olympus epi-fluorescence microscope equipped with a dual-bandpass filter allowing the simultaneous visualization of propidium iodide and fluorescein. Images were recorded using a SPOT Version 2.1.2 Camera (Diagnostic Instruments) and Image Pro Plus 4.1 software (Media Cybernetics).

Quantifying signal within and among chromosomes

Metaphase chromosomes hybridized with LINE Cper2 (which fluoresces green/yellow) were chosen in which the X, Y₂, and chromosome 1 (the largest autosome) could be unequivocally identified and did not overlap each other. Spreads were chosen according to the sharpness of chromosomal definition. Eleven such metaphase spreads hybridized to Cper2 were selected (six from TK104313 and five from TK104312). The Image Pro Plus 4.1 program was used to quantify the intensity of green signal along each of these three chromosomes (only a single chromosome 1 from each spread was measured to equalize the database). To estimate variation across the width and along the length of the chromosome, the signal was calculated along two separate lines drawn from telomere to telomere along the center of each chromatid. This program generated signal levels for bins along the length of the chromosome (e.g., typically well over 100 bins for chromosome 1, over 70 bins for the X, and over 35 bins for the Y₂), although the number of bins was lower in more contracted metaphase chromosomes than in less contracted ones. The background signal was measured and subtracted within each spread. The junction between the ancestral X and the translocated autosome was identified by the presence of a secondary constriction with reduced LINE signal (arrows in Fig. 2), and the X was then partitioned into one segment representing the original X (X_O) and one segment representing the translocated autosome (X_A).

For each chromosome (Fig. 2), the average intensity of green signal per unit length (bin) was determined for each of the two lines drawn through a chromosome, and the averages of both lines were themselves combined into a single average for the chromosome. Thus, each metaphase spread produced an average value for each of the four chromosome segments: 1_{*i*}, X_{O*i*}, X_{A*i*}, and Y_{2*i*} (where the subscript *i* indexes the particular metaphase in which the measurements were taken).

To consider whether the level of signal on X_O was different from that on chromosome 1, for example, the mean value for chromosome 1 was subtracted from the mean value for X_O in each metaphase spread, generating 11 differences: $D_i = X_{O_i} - 1_i$, $i = \{1, 2, \dots, 11\}$. The statistic $D/(sd/\sqrt{n})$ was treated as a *t* variable with $n - 1 = 10$ degrees of freedom (Snedecor and Cochran, 1980; where *sd* is the estimated standard deviation of the D_i). Because of between spread variation in the absolute intensity of hybridization, comparisons of absolute values are valid only within the same spread. By calculating differences between chromosomes within a metaphase spread and then applying statistics to those differences, any variation between spreads in the absolute intensity of hybridization is eliminated as a contributor to the differences between chromosomes.

Strongest area of signal within a karyotype

To determine which chromosome contained the strongest area of signal from the LINE probe, we identified the chromosomal position for the three areas with the strongest signal within each of the karyotypes. This was accomplished using the color-quenching capabilities of the Image Pro Plus 4.1 program. The green/yellow signal was quenched until no signal was visible on the monitor. By increasing and decreasing the intensity of the green signal, the order of appearance of the strongest areas of signal was determined within the karyotypes. For each karyotype, two researchers established scores that were then compared and verified.

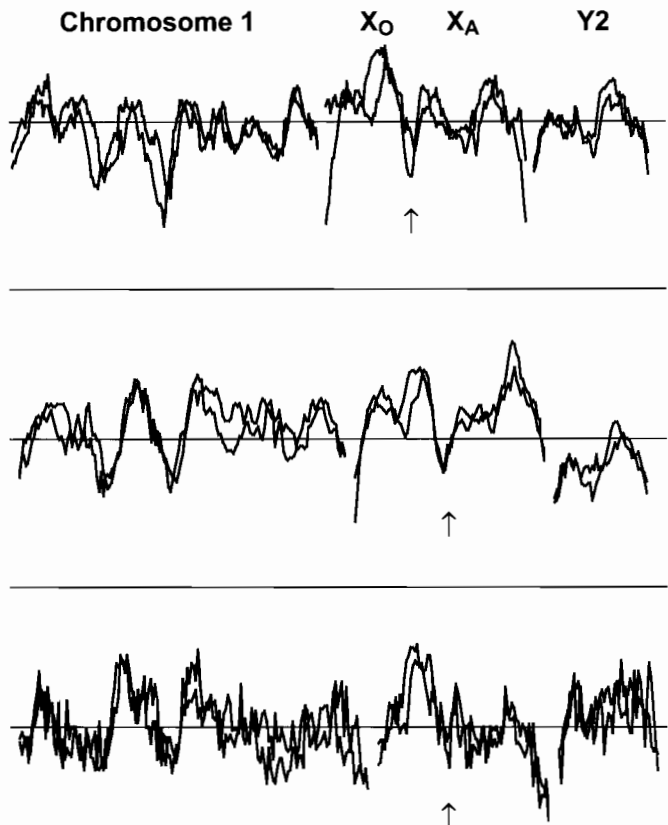


Fig. 2. Line graphs from three spreads (top, 13-9B; middle, 13-0A; bottom, 12-8A; see Table 1) showing variation in the four chromosomal segments that were scored for intensity of LINE signal using the Cper2 LINE probe. The two lines reflect scores recorded from each chromatid for the respective chromosomes. Chromosome 1 is the largest autosome, X_O is the original X, X_A is the autosome translocated to the X_O, and Y₂ is the homolog of X_A. The arrows identify the site of ribosomal genes that separate X_O from X_A. The horizontal line through each spread is the adjusted mean signal for that spread weighted equally for each chromosome. The line below each spread is the background hybridization level and shows that all detected signal is well above background.

Results

LINE-1 probe

When probed with a clone of a LINE isolated from *C. perspicillata*, the karyotypes of the two *C. brevicauda* males show signal on all chromosomes (Fig. 3). As reported for humans (Korenberg and Rykowski, 1988), the signal is nonrandom and varies in intensity along and among the chromosomes. For the largest autosomes, the strongest regions of signal appear to match the Q-bands observed in *C. perspicillata* by Tucker and Bickham (1989). A comparison was possible because the karyotype of *C. brevicauda* is essentially indistinguishable from that of *C. perspicillata*. As can be seen in Fig. 3, homologous chromosomes share a high level of banding order and intensity. Larger chromosomes have regions of higher intensity when compared to most of the smallest elements in the karyotype. Three pairs of the smallest chromosomes appear to have the weakest signal from an entire chromosome perspective; how-

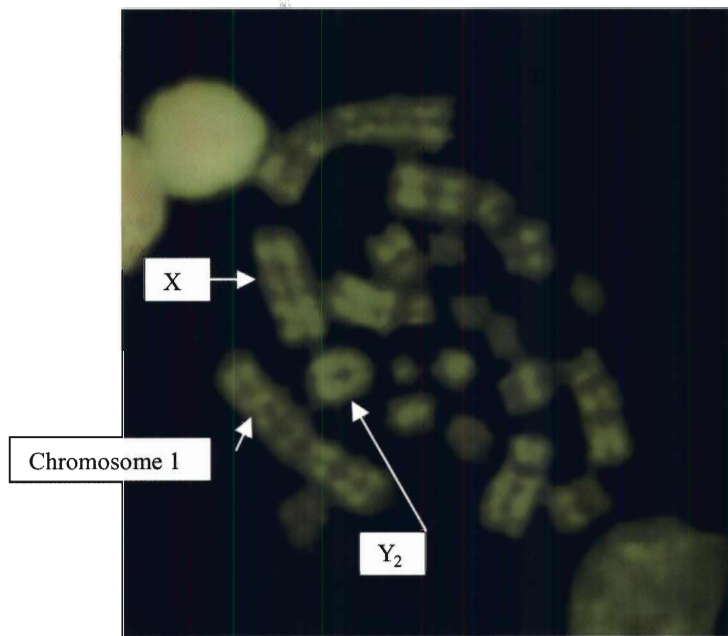


Fig. 3. FISH spread of a *Carollia brevicauda* male hybridized with the Cper2 LINE-1 probe.

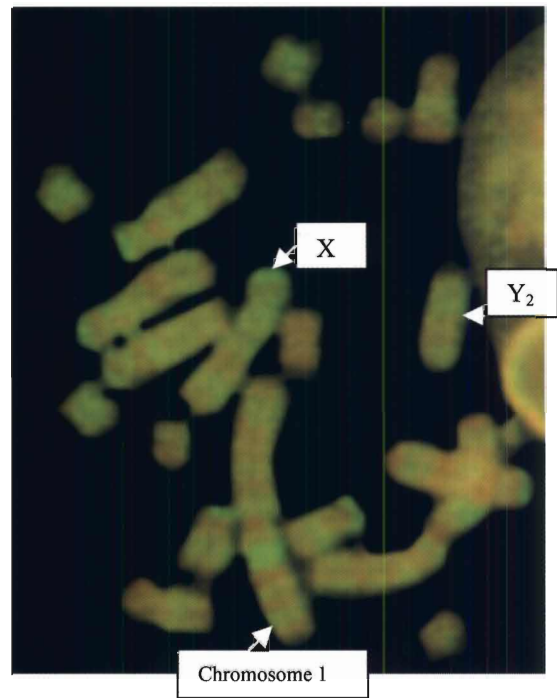


Fig. 4. FISH spread of a *Carollia brevicauda* male with a probe made from total genomic DNA from a male *C. brevicauda* individual.

ever, one small pair appears to have the signal typical of the larger chromosomes. The Y_1 (original Y), which is the smallest chromosome in the karyotype, has an intermediate level of signal relative to the other small chromosomes. Centromeric regions of most chromosomes appear to have little or no hybridization to the LINE probe. The secondary constriction present at the X_O - X_A boundary has the lowest level of signal for LINE probes on the long arm of the X_OX_A (Figs. 2 and 3).

Total genomic probe

Karyotypes hybridized with total genomic DNA isolated from *C. brevicauda* (Fig. 4) produced a similar pattern to that observed for LINES, with matching bands among the homologs. In general, the definition of bands on larger autosomes was sharper than on the smaller autosomes, and the areas identified as C-bands (Tucker and Bickham, 1989) also produced an intense signal with total genomic FISH. The size of the regions that showed minimal or no hybridization with the LINE probe appears to be smaller in the karyotypes hybridized with the total genomic probe.

Quantification of signal on chromosomes

The intensity of hybridization was quantified for 11 metaphase preparations hybridized with LINE Cper2 (Table 1). The X chromosome was divided into regions corresponding to the original X (X_O) and the translocated autosome (X_A). Per unit length, the hybridization signal of the X_O was significantly greater than of the three other chromosomes tested ($P < 0.0025$

for the X_O versus chromosome 1, $P < 0.005$ for X_O versus X_A , $P < 0.05$ for X_O versus Y_2 , and $P < 0.005$ for X_O versus the average of X_A and Y_2 ; all one-tailed t tests). As expected, the level of signal was not significantly different between the X_A and Y_2 ($P > 0.5$). Nor were any significant differences observed between chromosome 1 and either the X_A , Y_2 , or the average of X_A and Y_2 (all based on two-tailed t tests, since there was no a priori expectation of a difference either way).

Determination of the strongest area of signal

The above analysis was restricted to one representative of the largest autosome and the sex chromosomes. That analysis documents that the original X has the strongest average signal among X_O , X_A , Y_2 , and chromosome 1; it does not address, however, the question of whether or not there is another band or region in the entire karyotype with a stronger signal. To determine the chromosomal position of the strongest signal in the entire karyotype, we identified the three brightest regions in each spread. The strongest region of signal for 10 of the 11 spreads was as follows: in seven, the first signal to be detectable was on the X_O ; one initial signal was on the X_A ; one was adjacent to the centromere on the short arm of chromosome 1; and one was on the distal end of the long arm of a medium-sized subtelocentric chromosome. The strongest signal in the final spread was on the short arm of a medium-sized subtelocentric, but this region had two overlapping chromatids. Areas of overlapping chromatids give a stronger signal, which we interpret as being a technical problem. In this particular spread, we were

unable to determine which of several other areas had the second strongest signal, and, consequently, the results from this spread are not reported further.

The chromosomal location for the second strongest area of signal was adjacent to the centromere on the short arm of chromosome 1 (four spreads), the distal end of the long arm of a medium-sized subtelocentric (three spreads), and the X_A (two spreads). The third site to be visible was the most problematic to establish; but where it was possible, this site was on the distal end of the long arm of a medium-sized subtelocentric (four spreads), adjacent to the centromere on the short arm of chromosome 1 (one spread), and the Y₂ (one spread).

Although there is considerable variation in the level of signal among and within chromosomes, this method also reveals that the X_O is most often the strongest signal in the karyotype, followed by a band near the center of the largest autosomes and then a medium-sized subtelocentric chromosome. Strong signal is also present on the X_A, Y₂, and Y₁.

Discussion

Quantitative in situ hybridization

The data used here to estimate relative densities of LINES and other repetitive sequences on different chromosomes are collected from fluorescent in situ hybridization (FISH) with a biotin-labeled DNA probe. The signal is generated by fluorescein avidin/biotinylated anti-avidin reacting with the biotin-labeled probe. This method is commonly used to identify chromosome regions that contain repetitive DNA. We thus view our efforts as a specific test of the Lyon (1998a, b) model, as well as an exploration of potential difficulties in the quantitative inference of in situ hybridizations and how those difficulties may be overcome.

A central assumption behind our methods is that the density and accessibility of DNA per unit length of chromosome are similar across the chromosome segments we compare. Obviously, if two chromosomes with the same densities of sequences complementary to a probe contract at different rates, the more contracted chromosome will appear to have the higher density. A similar consideration applies to factors that affect access of the probe to the DNA. In our analysis, we used males, which ensured that the X examined for signal intensity was not inactivated which could result in a bias due to greater contraction associated with X chromosome inactivation. Because constitutive heterochromatin often does not have abundant copies of LINES, this could lower the score for LINE signal if the relative abundance of heterochromatin varies among chromosomes. This does not appear to explain the variance in our comparisons of chromosomes.

Our quantification of probe signal across multiple metaphase spreads from the same individual and several chromosomes within each metaphase spread reveals a substantial amount of noise that appears unrelated to the true variation in LINE content. Table 1 shows the level of between-metaphase variation, but within-metaphase variation is also evident from the fact that the Y₂ and X_A show large differences (in both directions) across different metaphase spreads from the same

Table 1. Average hybridization of LINE accumulation per unit length of chromosome^a

Spread	Chr 1	X _O	X _A	Y ₂
13-6A	85.89	116.57	114.81	84.26
12-7A	57.21	74.17	69.51	76.39
12-8A	60.28	70.85	54.61	65.59
12-9B	54.30	55.20	49.43	46.09
12-10A	60.72	69.72	60.71	63.94
12-11A	42.73	42.34	44.61	48.77
13-0A	67.42	75.45	75.52	52.38
13-7A	74.12	75.46	73.46	77.10
13-8A	69.39	83.01	75.99	84.50
13-9B	67.10	80.92	71.77	69.97
13-12C	37.42	47.26	41.69	48.35
Mean	61.51	71.91	66.56	65.21

^a Average hybridization intensity is shown for each of the 11 metaphase spreads analyzed. The numbers 12 and 13 identify the two male bats analyzed. Signal on the original X (X_O) and the attached autosome (X_A) are separated as described in the text. Units are arbitrary. Because of between-spread variation in hybridization intensity, valid comparisons can be made only among chromosomes within the same spread.

individual. This level of noise requires the use of at least moderately large samples combined with statistics based on differences among chromosomes from the same metaphase spread (rather than differences among pooled values from chromosomes across multiple metaphase spreads). This level of noise also warrants considerable replication of a pattern before the pattern should be accepted. Nonetheless, the empirical data revealed significant differences among chromosomes, and a similar degree of excess accumulation on the X (7% and 8%) for the two individuals examined.

Comparison of resolving power of probes

A comparison of the LINE-1 FISH and the total genomic FISH suggests that LINE distribution corresponds to most of the repetitive signal in the bat genome. No short interspersed nuclear elements (SINEs) have been described for bats, and information concerning the presence/absence of SINEs in the bat genome is needed to better understand the implications of the total genomic FISH observed for *Carollia*. Heterochromatic blocks appear only in the total genomic FISH, and are similar to C-bands for homologous elements in a given spread. These results suggest that FISH using LINES or total genomic DNA probes may provide adequate resolution for chromosomal identification while avoiding problems associated with G-banding species for which methods have not been clearly defined. The observation that total genomic FISH provides greater acuity of bands suggests that of the two probes, total genomic FISH may provide the greatest number of bands for analysis where chromosomal banding has application.

LINE distribution and failure to reject the Lyon repeat hypothesis

The Lyon model requires a higher density of LINES on the inactivated portion of the X than on chromosomal regions that do not undergo inactivation. Because the spread of XIST along

the X appears to be a *cis*-acting mechanism, her model does not preclude the existence of regions on other chromosomes that also have high densities of LINES. A LINE-dense region on a different chromosome would be too distant to attract XIST. Instead, the predictions of the model are local to the X chromosome and portions attached to the X: (1) autosomes attached to the X that escape inactivation should have a low density of LINES, and (2) autosomes attached to the X that undergo inactivation should have a high density of LINES similar to that observed in the ancestral region of the X itself. Lyon's model was derived from observations of *de novo* X-autosome translocations that collectively revealed these two properties. Likewise, further tests of this model are possible by investigating other X-autosome translocations.

All species in the genus *Carollia* have multiple sex chromosomes (X, Y₁, and Y₂; see Fredga [1970] for a review of the system and its taxonomic distribution), resulting from an X-autosome translocation. Based on the sequence divergence of the cytochrome b gene (Wright et al., 1999), the last common ancestor for all species in the genus *Carollia*, in which the X-autosome translocation is present, was estimated to have existed 7 million years ago. (One species, *C. castanea*, is polymorphic for this system [Patton and Gardner, 1971].) The juncture between the ancestral X and the translocated autosome is demarcated by a secondary constriction, which hybridizes to a ribosomal probe using FISH (Baker et al., 1992). Herein, we tested whether the original X stained more intensively with a probe for LINES than do other parts of the karyotype.

Results from our analysis indicate that the abundance of LINE signal on the X_A is not statistically different from the amount of signal on chromosome 1 or Y₂, but that the X_O has a statistically significant greater level of signal than is present on the X_A. If LINE abundance is critical to X-chromosome inactivation and X_A was statistically indistinguishable from the X_O in intensity of LINE signal, then an extension of the Lyon repeat hypothesis would predict that the X_A would accompany the original X in inactivation. However, the condition that the X_A has remained autosomal in its distribution of LINES would predict that the X_A should not be inactivated. Replication studies by Pathak et al. (1973) indicate that for the X and attached autosome, only the inactivated X_O chromosome replicates late in *Carollia* females and that the X_A attached to the late-replicating X_O replicates in the same timeframe as the other autosomes. These data are compatible with the hypothesis that the abundance of LINES on the X chromosome is critical to X inactivation. Therefore, the possibility that LINES act as booster elements for X inactivation remains viable.

In contrast to tests based on new X-autosome translocations, ancient translocations have a special advantage. LINES may accumulate in sex-linked regions for various reasons unrelated to XIST, so a several million year old X-autosome translocation may thus show a different pattern than a new one. The species studied here, *C. brevicauda*, carries an X-autosome translocation that appears to be as old as the genus. Given the timeframe of the translocation, much of the signal we are seeing with the LINE probe is most likely from elements that inserted subsequent to the translocation event. (See Casavant et al. [2000] for evidence that older elements may lose their ability to

be detected by FISH.) Thus a high density of LINES was likely maintained on X_O without a concomitant accumulation on X_A. The age of the translocation does not specifically enter into the test of Lyon's model, but older translocations may merely affect the opportunity for the model to be shown wrong if LINES accumulate on the attached autosome for reasons unrelated to the Lyon model.

The secondary constriction, which is thought to consist of a region of tandemly repeated ribosomal genes, possibly may provide a deterrent to XIST spreading, similar to the observation by Duthie et al. (1999) that large blocks of heterochromatin are a barrier to XIST spreading in rodents. Whether other cases of an autosomal translocation to the X have similar physical barriers that could play a role in differential inactivation of the true X is important to determine.

Since the density of LINES on these chromosomes may be affected by a large number of processes, only one of which involves their possible role in X inactivation, our test of the Lyon model (1998a) is one-sided. A high density of LINES on the early replicating autosomal portion of the X warrants the model's rejection and thus constitutes a strong result, whereas a low density of LINES is consistent with the model but admits many alternatives, and thus is a weak result. Ideally, this type of test should be performed on many independently evolved X-autosome translocations of different ages. As little as one clear exception to the expected pattern would indicate that LINES are not the sole mechanism of XIST spreading. Our paper provides one such test. Although more tests of this model are needed, our paper may be viewed as an illustration of the procedures and limitations of such tests based on *in situ* hybridization.

Our results are consistent with the mechanisms previously proposed to explain X-chromosome accumulation of LINES. In particular, if LINES are removed more rapidly from recombining regions of the genome by ectopic excision (Langley et al., 1989), X_A would be expected to undergo recombination in all individuals, whereas X_O would undergo recombination only in females. Thus elements would be removed more rapidly from X_A and would accumulate on X_O. Similarly, if LINES are inserting preferentially into late-replicating regions of the genome, they would be expected to accumulate on X_O, which is subject to late replication during X inactivation, but not on X_A, which escapes inactivation (Pathak et al., 1973). Interestingly, Y₁—the original Y—would also be expected to undergo late replication, but it does not show the strong accumulation of LINES seen on other mammalian Y chromosomes.

Acknowledgements

We thank Federico Hoffmann, Brenda Rodgers, LuAnn Scott, and Jeff Wickliffe for assistance.

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