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Steinmann-Zwicky, M; Nöthiger, R

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Abstract

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A Small Region on the X Chromosome of Drosophila Regulates a Key Gene That Controls Sex Determination and Dosage Compensation

Monica Steinmann-Zwicky and Rolf Nöthiger
Zoological Institute
University of Zurich
Winterthurerstrasse 190
CH-8057 Zurich, Switzerland

Summary

In Drosophila, flies with two X chromosomes are females, with one X chromosome, males. We investigated the presence of sex determining factors on the X chromosome by constructing genotypes with one X and various X-chromosomal duplications. We found that female determining factors are not evenly distributed along the X chromosome as had been previously postulated. A distal duplication covering 35% of the X chromosome promotes female differentiation, a much larger proximal duplication of 60% results in male differentiation. The strong feminizing effect of distal duplications originates from a small segment that, when present in two doses, activates *Sxl*, a key gene for sex determination and dosage compensation. Our results suggest that *Sxl* can be activated to intermediate levels.

Introduction

Sex differentiation is an important developmental event in the life of an animal. Understanding the genetic regulation of this early decision might give insights into the mechanisms by which a choice is made between two alternative developmental pathways in general. Drosophila melanogaster with its wealth of genetic tools provides an ideal system to investigate the genetic pathway responsible for sex determination. Yet, in spite of many years of research, the primary signal that implements female vs. male development is still not understood. We know little more than that the ratio of X chromosomes (X) to sets of autosomes (A) provides a genetic signal for two closely linked processes, sex determination and dosage compensation. An X:A ratio of 1.0 (XX; AA) triggers female differentiation, a ratio of 0.5 (XY; AA) leads to male development. Dosage compensation provides for equal amounts of X-chromosomal gene products in the two sexes, XX and XY. This is achieved by regulating the transcriptional activity of X-linked genes in such a way that it is high in males and low in females (for reviews see Lucchesi, 1977; Stewart and Merriam, 1980; Baker and Belote, 1983).

According to the theory of genic balance (Bridges, 1921), the X chromosomes carry female determinants, the autosomes, male determinants. The "weight" of these factors was assumed to be such that two X chromosomes outweigh two sets of autosomes, but two sets of autosomes outweigh one X. A strong argument for this view derived from animals with two X chromosomes and three sets of autosomes (XX; AAA), which displayed a mosaic phenotype of male and female cells. Addition of X-chromosomal duplications to these so-called triploid intersexes led to a feminization of their phenotype, deletions to a masculinization. The effect was roughly proportional to the size of the duplication or deletion, but independent of what part of the X chromosome had been added or deleted (Dobzhansky and Schultz, 1934; Pipkin, 1940). These results suggested a purely quantitative effect achieved by many female determining factors scattered along the X chromosome. Attempts to localize major female determining genes in diploid animals failed. When small duplications of various regions of the X chromosome were added to males, none produced any shift toward femaleness. Similarly, females carrying deficiencies on one of their X chromosomes never showed any male characteristics (Patterson et al., 1935; 1937; Patterson and Stone, 1938; reviewed by Pasztor, 1976; Laugé, 1980; Baker and Belote, 1983).

The work referred to suffers from certain shortcomings. First, the crucial experiments that established the presence of numerous female determining factors scattered over the entire X chromosome were done with triploid intersexes (XX; AAA) as a reference. The mosaic phenotype of these animals, however, is inherently variable and susceptible to even slight changes in genetic and environmental factors (Cline, 1983). Second, in diploid animals only short duplications or deficiencies, and only an incomplete sample, were studied since aneuploidy for larger parts of the X chromosome is lethal.

We therefore reexamined the sex determining effect of various regions of the X chromosome in diploid animals and included aspects of dosage compensation in our analysis. The diploid condition is more stable and should provide a more reliable reference than XX; AAA. We investigated the sexual phenotype of cells with two sets of autosomes and an intermediate X:A ratio of between 0.5 and 1.0 by providing the animals with more than one, but less than two, X chromosomes. The problem of zygotic lethality of such genotypes was overcome by producing genetically mosaic animals that displayed the desired genotype in clones whose sexual phenotype could then be assessed under the compound microscope. This is possible because lethal genotypes are frequently viable in clones of mosaic animals (Ripoll, 1977; 1980) and because the sexual phenotype is expressed cell-autonomously in clones (Stern and Hannah, 1950).

The results of our study reveal that major female determining factors are located distally on the X chromosome. We could define two distal elements, one being the previously described gene Sex-lethal (*Sxl*), which controls sex determination and dosage compensation (Cline, 1978; Lucchesi and Skripsky, 1981). The other element, chromosomal region 3E8 to 4F11, or the even smaller segment 3F3 to 4B1, is involved in the activation of *Sxl*. We also present indirect evidence that *Sxl* can be activated to intermediate levels.

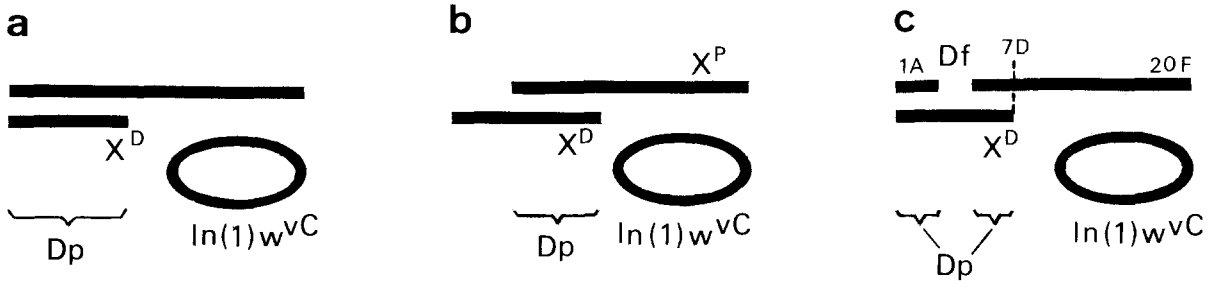
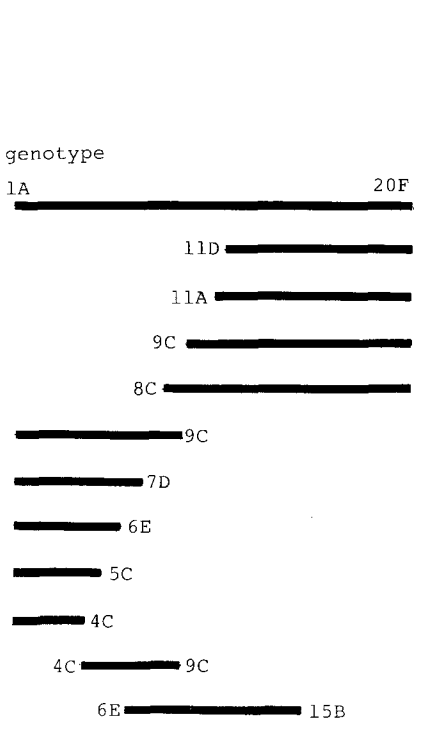


Figure 1. Production of Genotypes with Intermediate X:A Ratios

Flies with two X chromosomes and a duplication (X/X/Dp) were constructed. Such animals are perfectly viable females although their fertility is reduced. One of the X chromosomes was the unstable ring X chromosome, *In(1)w^{vC}*, which is occasionally lost during early cleavage. This produces clones of genotype X/Dp. (a) Production of distal duplications with fragments of the distal part of the X/X^{D}); in the same way proximal duplications with X^{P} were constructed; (b) production of interstitial duplications using overlapping X^{D} and X^{P} fragments; (c) females of the genotype *Df(1)/In(1)w^{vC}/Dp(1A to 7D)* were constructed. The deficiency (Df) is located within the region covered by the duplication, thus reducing the size of the duplicated area. If the unstable ring chromosome is lost, a clone of genotype *Df(1)/Dp(1A to 7D)* arises whose sex then is assessed.



n	head	thorax	female: abdomen	female: anal plates	female: genitalia	female: first leg	male: abdomen	male: anal plates	male: genitalia	male: first leg	sex
16	3	5					42	10	10	4	♂
25	1						40	2	4		♂
43	1	2					48	2	3	1	♂
24		1	1				31	2	3		♂
148			105	3	1						♀
124			104				1				♀
122	14	18	98				69	12	14	11	♀♂
83	30	52	31				164	37	34	29	♀♂
7	2	6					9	3	3	5	♂
34	18	34	3				74	40	41	28	(♀)♂
21			2				19				(♀)♂

Figure 2. Distribution and Sex of Clones of Genotypes with Intermediate X:A Ratios

On the left, the duplicated area is schematically represented by a line for each tested genotype. The X chromosome is divided into 20 numbered units each of which is subdivided into six lettered units from A to F (Bridges, 1938). The sexual phenotype is summarily represented by a symbol in the far-right column (sex). The column labeled n shows the number of mosaic flies analyzed. Left and right halves of each fly were separately scored for clones in head, thorax, foreleg (sex comb region), anal plates, genitalia, tergite 5, tergite 6, tergite 7, and sternite 7. The number of tergites and sternites with tissue of genotype X/Dp was then taken together and listed under abdomen.

Results

The sexual phenotype of cells with intermediate X:A ratios was analyzed. Since aneuploidy for larger parts of the X chromosome results in a significant imbalance of gene products, which causes lethality, the desired genotypes had to be scored in clones of mosaic flies produced as shown in Figure 1. A summary of the main results is presented in Figure 2, which shows the various duplications used. The legend explains the cytological nomenclature for the X chromosome. The terms proximal and distal designate the position of chromosomal sites or regions relative to the centromere that is located to the right of region 20F.

Sex of Aneuploid Genotypes

Proximal duplications led to a male phenotype (Figures 2 and 3a–3c). Sex combs, male genitalia, male analia, and male pigmentation on tergites 5 and 6 were found. Sternites 7 as well as tergites 7 were missing. The highest X:A ratio tested was 0.81, namely a duplication from region 8C to the centromere. Thus, 11.5 of the 20 units correspond-

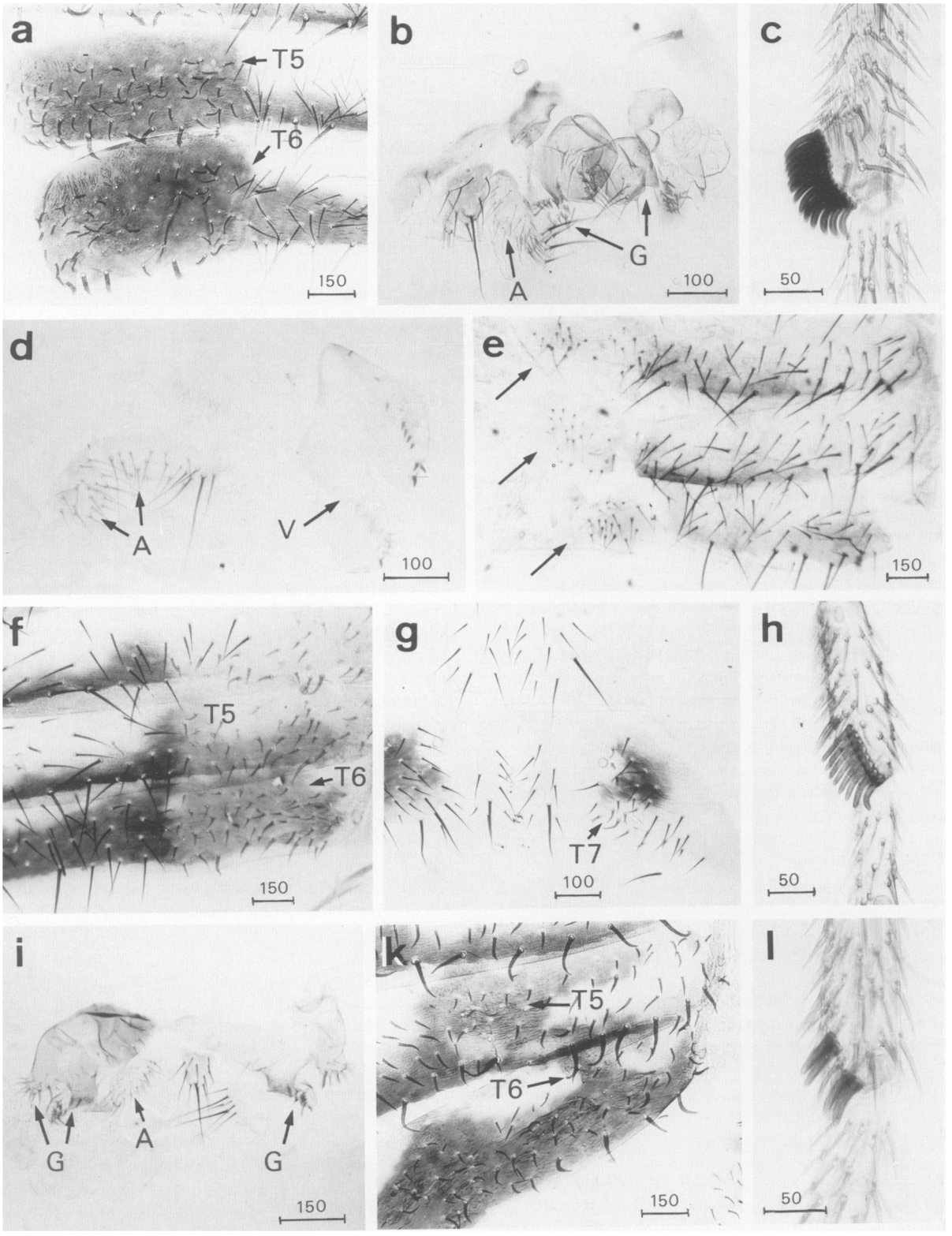


Figure 3. Photographs of Aneuploid Clones and Animals

Numbers above bars are microns. (a) *y w sn/Dp(9C to 20F)* (X^P of B26), tergite 5 (T5) and tergite 6 (T6) show male pigmentation, clone marked with *sn*; (b) *y f/Dp(8C to 20F)* (X^P of J8), male genitalia (G) and anal plate (A), clone marked with *y*; (c) *y w sn/Dp(11D to 20F)* (X^P of B39), male sex comb; (d) *y w sn/Dp(1A to 9C)* (X^D of B26), female vaginal plate (V) and anal plates (A), clone marked with *y*; (e) *y w sn/Dp (1A to 9C)* (X^D of B26), female, but poorly developed tergites (arrows), clone marked with *y*; (f–i) *y w f/Dp(1A to 6E)* (X^D of 149), clones marked with *y f*; (f) female pigmentation on tergite 5 (T5) and male pigmentation on tergite 6 (T6), (g) female tergite 7 (T7), (h) male sex comb, (i) male genitalia (G) and male anal plate (A). (k, l) Nonmosaic animals of genotype *y w* Sxl^f f^{3ea}*/Df(1)HF366 (3E8 to 5A7)*; (k) partial male pigmentation on tergite 5 (T5) and tergite 6 (T6); (l) four male sex comb teeth with an interspersed slender female bristle.

ing to some 60% of the euchromatin of the X chromosome were duplicated, and yet this genotype resulted in male development.

Large distal duplications from 1A to 9C, but also from 1A to 7D, led to a female phenotype (Figures 2 and 3d). The lowest X:A ratio that gave female differentiation was 0.67. This represents a genotype in which only seven and a half or some 35% of the 20 euchromatic units of the X chromosome are duplicated. The smaller duplications up to 6E or 5C led to an intersexual phenotype. In these flies, the terminalia and the sex combs were always male, whereas the tergites and sternites were either female or male (Figures 2 and 3f–3i).

Tissue with the interstitial duplication 4C to 9C, as well as 6E to 15B, displayed intersexuality inasmuch as some clones in the abdomen were female (3 out of 77 and 2 out of 21, respectively). All other clones were male, both in tissue deriving from imaginal discs and in the tergites (Figure 2).

These results show that sex is not determined by a mere quantitative effect of X versus autosomal chromosome material (X:A ratio). Major sex determining factors must be present distally on the X chromosome.

Viability of Aneuploid Genotypes

When genotypes with intermediate X:A ratios that led to lethality were studied in clones, we noticed differences with respect to the size of the clones, their location, and the quality of the structures differentiated by them. These parameters are important, since, as discussed later, they can be used as an indirect measure of dosage compensation. The distribution of clones in head, thorax, and abdomen is shown in Figure 2.

Proximal duplications were well tolerated. Tissue of genotype *X/Dp(11D to 20F)* developed in all body regions of the flies; with still larger duplications, clones were frequently found in the abdomen, but more rarely in head and thorax. The structures produced by these clones were always well differentiated.

Distal duplications, at most up to 6E, also allowed ubiquitous and normal differentiation of tissue. In contrast, clones with larger distal duplications of up to 7D or 9C only survived in the abdomen, and no mosaic structures deriving from cephalic and thoracic imaginal discs developed. Furthermore, tergites and sternites differentiated poorly and the size of the structures formed was usually reduced (Figure 3e).

In general, we observed a positive correlation between the size of a duplication and its detrimental effect, although exceptions to this rule occurred. An even better correlation, however, exists between the sex and viability of clones produced by a given genotype. It is striking that the most severely affected genotypes are those that differentiate only female tissue. Where male or intersexual clones were formed, viability was much better.

Localization and Identification of Functions Essential for Female Development

The genotype with an X and a duplication for 1A to 7D developed female structures. We tested whether within 1A to 7D a smaller region could be defined, of which two doses are necessary to promote female differentiation. If such a region existed, then hemizygosity for this region, achieved as explained in Figure 1c, should shift the sexual phenotype of developing tissue from female to intersexual or to male. The results obtained with a series of twelve distal deficiencies are given in Figure 4a. In nine of the genotypes tested, only female clones could be found, and only on sternites and tergites. Intersexual differentiation ensued when the duplication 1A to 7D was combined with one of the three deficiencies from 6E4 to 7A6, from 3E8 to 5A7, or from 3E8 to 4F11, thus defining two regions with functions or genes essential for female development. In all three genotypes, clones were found in all tissues, and the latter two gave rise to whole flies. These flies were male, some of which (4 out of 21, and 2 out of 5, respectively) had a few bristles on an additional tergite that, however, did not show the characteristics of a female tergite 7.

Region 6E4 to 7A6, which was shown to contain one or several genes essential for female development, includes *Sxl*, a gene known to be instrumental in sex determination and dosage compensation (Cline, 1978; 1979; Lucchesi and Skripsky, 1981). To test whether *Sxl* is responsible for the feminizing effect of this region, we combined duplication 1A to 9C with an entire X chromosome carrying Sxl^f, a recessive mutation that eliminates the Sxl^+ function (Cline, 1978). This genotype (see Figure 7, genotype F) survived in clones and differentiated an intersexual phenotype, namely male terminalia and sex combs, whereas tergites and sternites could be of either sex. Clones were found in all structures of the fly (Figure 4b). Thus, two doses of Sxl^+ are needed in the genotype *X/Dp(1A to 9C)* (see Figure 7, genotype E) for female differentiation.

Interaction between *Sxl* and Region 3E8 to 4F11

In the previous section, the gene *Sxl* and region 3E8 to 4F11 were shown to play an essential role in female development. Both are needed in two doses in the genotype *X/Dp(1A to 9C)* or *X/Dp(1A to 7D)* for female differentiation. Yet, in a fly with two X chromosomes, the mutation Sxl^f or a deficiency for 3E8 to 4F11 in a heterozygous condition are recessive, allowing normal female development (see Figure 7b, genotypes G, J). Since, as our results suggest, both functions are needed for female development, and additive effect might be expected if Sxl^f and a deficiency for 3E8 to 4F11 were simultaneously present in heterozygous condition in flies with two X chromosomes. Genotype Sxl^f/*Df(3E8 to 4F11)* (see Figure 7, genotype H) an Sxl^f/*Df(3E8 to 5A7)* differentiated as sexual mosaics that were essentially female with some male characteristics, such as sex combs (1 to 7 teeth), male pigmentation on tergites 5 or 6, and occasionally male anal and genital structures (Table 1 and Figures 3k, 3l). The degree of masculinization and the viability of the flies depended on temperature. At 25°C the viability was good and only half of the flies showed male characteristics. At 21°C, viability was poor and most of the flies were partially masculinized. At 18°C no flies survived. Control experiments were performed to check whether simply a quantitative lack of X chromosome material was responsible for the sexual

		n	head	thorax	female abdomen	female anal plates	female genitalia	female first leg	male abdomen	male anal plates	male genitalia	male first leg
a												
	•••1B10	28			27							
	1E5••••••2B15	27			59							
	2C1•••••••••3C5	22			41							
	2E1••••••3C2	33			56							
	3C6•••3D2	30			41		1					
	3C11•••3E4	30			52							
	3E8•••••••••4F11	28	10	10	31				33	10	10	8
	3E8••••••••••••5A7	28	39	42	14				98	46	46	41
	4B1•••••••4F1	29			52							
	5A8••••5C5	27			60							
	5C5••5D5	22			37							
	6E4••••7A6	82	2	3	85				6	4	4	3
b	Sxl^f / Dp (1A to 9C)	18	6	3	7				2	3	3	2

Figure 4. Functions Essential for Female Development

(a) Distribution and sex of clones of the genotype *Df(1)/Dp(1A to 7D)*. The dotted lines represent the size of each of the 12 deficiencies tested. Two regions, 3E8 to 4F11 and 6E4 to 7A6, need to be present in two doses for female differentiation to ensue.

(b) The last genotype tested, *Sxl^f/Dp(1A to 9C)* is intersexual. This points to *Sxl^+*, located in 6F/7A, as the gene responsible for the masculinization of genotype *Df(6E4 to 7A6)/Dp(1A to 7D)* in Figure 4a. The male structures whose occurrence identifies the chromosomal regions that are crucial for female development are boxed.

Table 1. Interaction between *Sxl* and Region 3E8 to 4F11

Genotype Df / *Sxl^+* / *Sxl^f*	Number of Flies Analyzed	Flies with Sex Comb Teeth	Flies with Partially Male Pigmentation on Tergites 5 and 6	Flies with Partially Male Anal Plates	Flies with Some Male Genitalia	Females without Any Male Trait
Df (3E8 to 4F11)/Sxl^f 21°C	20	11	3	4	1	?
Df (3E8 to 5A7)/Sxl^f 21°C	122	86	84	17	16	9
Df (3E8 to 4F11)/Sxl^f 25°C	40	8	13	0	2	21
Df (3E8 to 5A7)/Sxl^f 25°C	22	8	7	0	0	10

At 21°C the flies were poorly viable, especially genotype *Df (3E8 to 4F11)/Sxl^f*, for which 15 out of 20 flies died as pharate adults. In animals extracted from the pupal case it is not yet possible to see male pigmentation on tergites and as a consequence we were not able to score the number of flies without any male trait. At 25°C both genotypes were fully viable; at 18°C both genotypes were lethal.
Df, deficiency.

transformations. All other deficiencies presented in Figure 4 were also combined with the mutation *Sxl^f*. They gave rise to normal females, except that 10 out of 32 flies of genotype *Sxl^f/Df(3C6 to 3D2)* showed small pigmented patches on tergites 5 or 6, but no other male characteristics. Thus, we conclude that *Sxl* interacts specifically with region 3E8 to 4F11.

Two Doses of Region 3F3 to 4B1 Lead to Lethality in Males Because Their *Sxl* Gene Becomes Activated

Activity of the gene *Sxl^+* is essential for female development and lethal to males (Cline, 1978). The product triggers female sexual differentiation and low transcription rate of X-chromosomal genes (Lucchesi and Skripsky, 1981; Cline, 1983). We would therefore expect that males carrying a particular duplication might be lethal if this duplication activated *Sxl*, which would then implement a low transcription rate of the single X chromosome. Using X–Y translocations with different breakpoints, we could duplicate almost any region of the X chromosome (Figure 5). Such genotypes with one X chromosome and one of the duplications listed in Figure 5 developed as males that

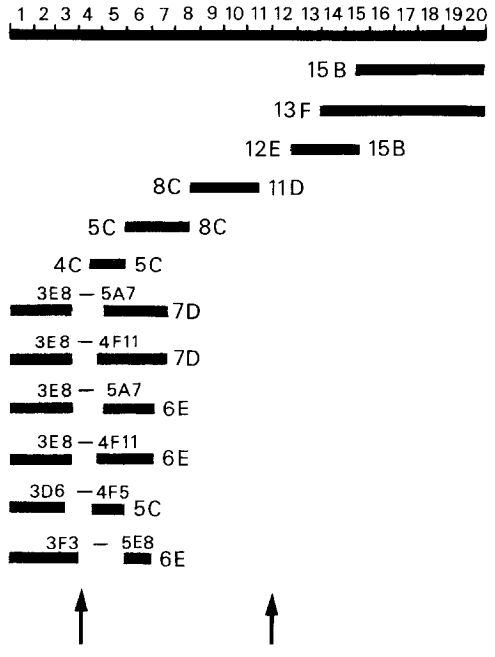


Figure 5. Genotypes with One X Chromosome and a Duplication of X Material Viable in Males

The lines represent one whole X chromosome (1–20) and the duplicated area below. The first two lines show proximal duplications. The next four lines represent interstitial duplications. The last six genotypes carry a deficiency on the X chromosome and a distal duplication. To simplify the representation we drew two lines, as if the deficiency was on the distal duplication. The arrows point to the two regions 11D to 12E and 3F3 to 4C, which when duplicated were lethal in males. Since the genotype *Df(4B1 to 4F1)/Dp(1A to 7D)* that duplicates 1A to 4B1 was not viable (Figure 4), the distal region can be narrowed down to 3F3 to 4B1.

were either viable or died very late as pharate adults. We found, however, two regions that when duplicated caused early lethality, namely 3F3 to 4C and 11D to 12E. Stewart and Merriam (1973) also identified two regions on the X chromosome that could not be duplicated in males, namely 3A to 3E and 11D to 12E. They themselves question the results for 3A to 3E, since it is possible to duplicate it using smaller duplications. The authors used the same translocation, *T(X;Y)B29*, as we did. But we determined its breakpoint to be in 4C instead of in 3E (Figure 6). Thus, the region that Stewart and Merriam could not duplicate extends from 3A to 4C, which includes the region 3F3 to 4C that we defined by the present experiments. Since the genotype *Df(4B1 to 4F1)/Dp(1A to 7D)* is not viable (Figure 4), we conclude that a duplication for 1A to 4B1 causes lethality. Thus, the region that causes lethality when duplicated in males can be narrowed down to 3F3 to 4B1.

As we reasoned before, a duplication for region 3F3 to 4B1 might activate Sxl^+, and the resulting low transcription rate then would cause lethality of these males. To test this hypothesis, we constructed genotypes with one X chromosome carrying the mutation Sxl^f and a distal duplication from 1A to 4C (Figure 7, genotype B), and compared them to controls with Sxl^+ (Figure 7, genotype A). No flies of the control genotype A survived, whereas genotype B with Sxl^f gave viable males. In these latter flies, no Sxl^+ product can be made and the transcription rate of the X-linked genes will be high allowing better survival of the aneuploid males. A second experiment confirmed this interpretation. A small distal duplication, *Dp(3C2 to 5A1)*, is viable in males mutant for Sxl^f but lethal in males with Sxl^+. We conclude that two doses of region 3F3 to 4B1 activate the gene Sxl^+, which results in a low rate of transcription of the X chromosome and hence early death of animals with essentially a single X chromosome.

Discussion

The interpretation of our results requires some familiarity with sex determination and dosage compensation in *Drosophila*. The ratio of X chromosomes to sets of autosomes (X:A) is the primary genetic signal for both processes (Bridges, 1921; 1925; Steinmann-Zwicky and Nöthiger, 1985). This quantitative signal is taken up by the key gene *Sxl* (Sex-lethal) whose state of activity then determines the sexual pathway and the rate of transcription of the X chromosome (or chromosomes) (Cline, 1978; 1979; 1983; Lucchesi and Skripsky, 1981). Downstream of *Sxl*, the two processes are under separate genetic control. A small number of regulatory genes determines the sexual pathway, e.g. *tra* or *dsx*, and another set of genes governs the rate of transcription of the X chromosomes, e.g. *mle* or *msl-1* (reviewed by Baker and Belote, 1983). Sex determination and dosage compensation can thus be uncoupled by mutations in these genes.

The state of activity of *Sxl* is set early in development, around blastoderm formation, and is later maintained independently of the X:A ratio (Sánchez and Nöthiger, 1983; Cline, 1984). For correct X-chromosomal transcription and sex determination, the product of Sxl^+ is needed in flies with two X chromosomes, and it must be absent from flies with one X chromosome. Mutations in *Sxl* therefore act as sex-specific lethals and simultaneously lead to sexual transformation (Cline, 1978; 1979; Sánchez and Nöthiger, 1982). A recessive allele, Sxl^f, corresponding to a loss of function, leads to hyperactivity of the X chromosomes which is lethal in XX animals; it also results in male transformation of XX cells (Cline, 1979; Sánchez and Nöthiger, 1982). A dominant allele, Sxl^{M1}, corresponding to a constitutive expression of the gene, causes a low transcription rate of the X chromosome which is lethal in XY or XO animals; the mutation also produces a female transformation of XO cells (Cline, 1979). In this study, we therefore assumed that the *Sxl* gene was active whenever some female structures were formed.

A Small Distal Region on the X Chromosome Regulates *Sxl*

Earlier work with triploid intersexes led to the conclusion that the X chromosome harbors numerous female determining factors that are more or less evenly distributed over the entire chromosome (reviewed by Pasztor, 1976; Laugé, 1980). Our results now show that distal duplications have a much stronger feminizing effect than proximal duplications (Figure 2). Using different duplications and deficiencies, the strong feminizing effect of the distal part of the X could be assigned to two regions, 3E8 to

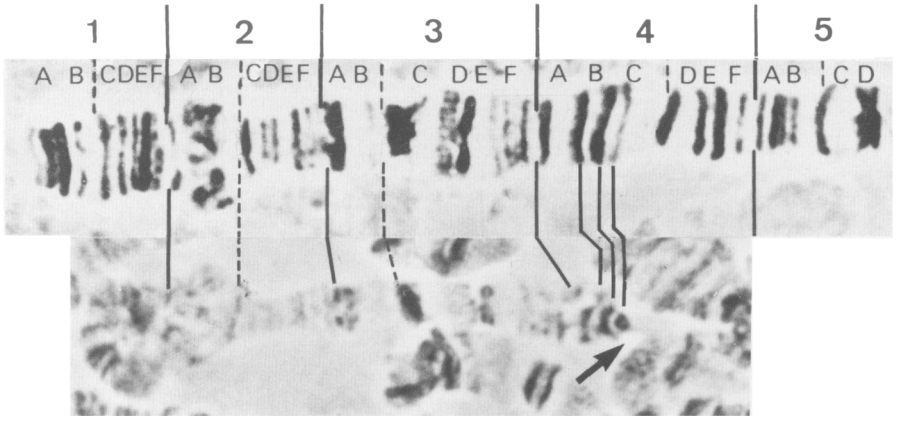


Figure 6. Salivary Gland Squash of a Male Larva Carrying the Translocation T(X;Y)B29

The lower photograph shows the distal element X^D. The breakpoint on the X chromosome is in 4C (arrow). Reference chromosome (upper photograph) modified from Lefevre (1976).

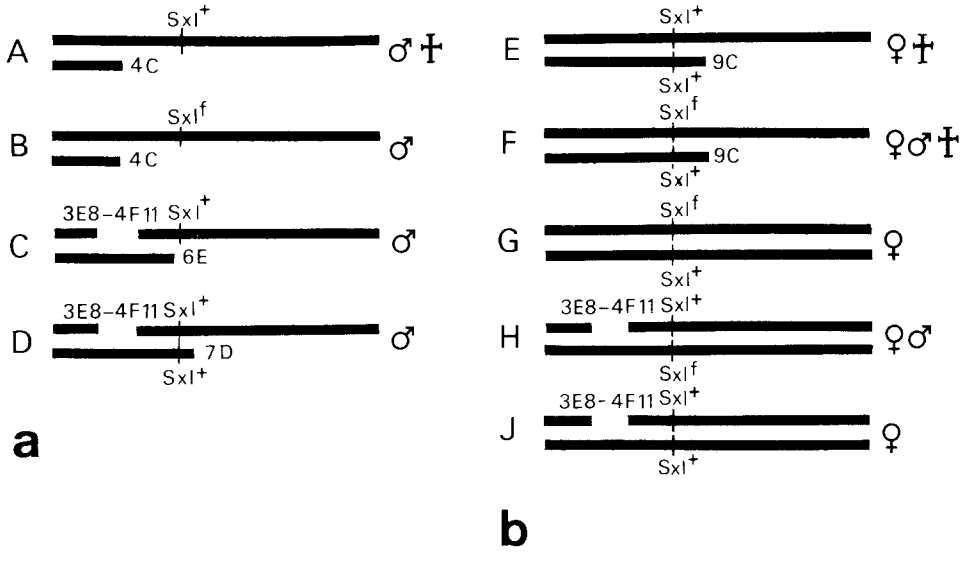


Figure 7. Representation of Selected Genotypes

Indicated are the breakpoints on the chromosomal map of Bridges (1938), the allelic state at the *Sxl*-locus, the sexual phenotype (♂ male; ♀, female; ♀♂, intersexual); † denotes genotypes that cause early lethality for whole animals, but survive in clones; interrupted chromosome, *Df(3E8 to 4F11)*. Sxl^f is a lack-of-function mutation.

(a) Four genotypes whose phenotypes show that two doses of a distal region activate Sxl^+ (see Discussion). All four genotypes suffer from imbalance of gene products. If Sxl^+ is active, as in A, the X-chromosomal genes are transcribed at a low rate which results in a deficit of their products relative to those of the autosomal genes. If *Sxl* is mutant (B) or not activated (C, D), the X chromosomes become hyperactive so that the balance of X-chromosomal to autosomal gene products is improved which allows a better survival (genotypes B, C, D vs. A). Irrespective of the level of activity of the X chromosomes, the products of genes within the duplication are twice as abundant as the products of genes outside of the duplication (Maroni and Lucchesi, 1980). Due to this imbalance, animals of genotypes B, C, or D are not fully viable; they usually die as adult males shortly after emergence, or as pharate adults.

(b) Five genotypes used to argue that Sxl^+ can be activated to intermediate levels, not just "on" or "off" (see Discussion).

4F11, and 6E4 to 7A6. Within 6E4 to 7A6, we identified the gene *Sxl* as being responsible for the feminizing effect of this region. Two wild-type copies of this gene must be present if aneuploid genotypes, such as the ones produced in our experiments, are to follow the female pathway. Within 3E8 to 4F11, we defined a much smaller region, 3F3 to 4B1, that cannot be duplicated in males because two doses of it activate the *Sxl* gene. Animals with one X chromosome and a duplication of this region, e.g. *X, Sxl^+/Dp(3C2 to 5A1)* or *X, Sxl^+/Dp(1A to 4C)*, die early in development, but when they carry the recessive mutation Sxl^f instead of Sxl^+ on their X chromosome, they survive to the adult stage (Figure 7, genotypes A, B). We conclude that an active Sxl^+ gene caused the lethality of the genotypes with *X, Sxl^+* by lowering the transcriptional activity of the X chromosome. The next two genotypes (C, D in Figure 7), which carry a deficiency for 3E8 to 4F11 and a large distal duplication, also developed to males that died as adults shortly after emerging, as did genotype B. Thus, these aneuploid animals with zero, one, or two Sxl^+ genes reach the same late stage. This indicates that the Sxl^+ genes in genotypes C and D were not activated,

and points out the critical role of the chromosomal region 3E8 to 4F11 which is present in only one dose in genotypes C and D.

The impact of the newly defined chromosomal region on the activation of *Sxl*$^+$ is further illustrated by a comparison of the genotypes *X/Sxl*^{f} and *X, Df(3E8 to 4F11)/Sxl*^{f} (G and H in Figure 7b). Both have one *Sxl*$^+$ gene and about the same X:A ratio of ~1.0, but they differ in having either two or one dose of region 3E8 to 4F11. Whereas genotype G is a normal female, genotype H develops an intersexual phenotype, indicating that the single *Sxl*$^+$ gene is sufficiently activated only when region 3E8 to 4F11 is present in two doses. Our experiments reveal the crucial role that region 3E8 to 4F11, and within it 3F3 to 4B1, plays in the activation of *Sxl*. The proximal half of the X chromosome, however, must also contain some minor female determining factors whose presence becomes apparent when we compare genotype F, which leads to intersexuality, with genotype G (Figure 7b), which produces a normal female. Both of these genotypes carry a single intact *Sxl*$^+$ gene that, however, is sufficiently activated to promote female differentiation in all cells only when the proximal part of the X is also present. One of these female determining factors is probably the newly discovered gene sisterless (*sis*, located in 10B), which also appears to be involved in the activation of *Sxl* (Cline, 1984).

Our experiments revealed a distal region on the X chromosome that causes lethality when duplicated in males. The lethal effect of a particular distal duplication was noticed before by Stewart and Merriam (1973) and even earlier by Patterson et al. (1937). The former, however, obtained conflicting results due to an error in determining the breakpoint of the translocation *T(X;Y)B29* (see Results). The latter had available only a limited number of genetic markers giving only a rough estimation of the size and extent of the duplication; furthermore, since escapers occurred (less than 1% and only in some of the experiments), they considered the genotype as viable. We now identify the chromosomal segment 3F3 to 4B1 as the region of which two doses lead to lethality in males, and we furthermore show that this lethality results from activation of *Sxl*$^+$.

At this point, the question arises why clones of genotype *X, Sxl*$^+$*/Dp(1A to 4C)* (A in Figure 7) differentiate male characteristics (Figure 2) even though their *Sxl*$^+$ gene has been activated. We will discuss this problem in the next section.

The Gene *Sxl*$^+$ Can Be Activated to Intermediate Levels

In euploid flies, sex and dosage compensation are strictly correlated. Animals with two X chromosomes are females and transcribe their Xs at a low rate; animals with one X chromosome are males and transcribe their single X at a high rate. Sex and dosage compensation are implemented by the X:A ratio and are under the common control of *Sxl*. Normally, the X:A signal is unequivocal, either 1.0 in females or 0.5 in males, and all cells of an animal respond in the same way. In our aneuploid animals, however, the X:A signal is ambiguous. When this led to an intersexual phenotype, the result was a mosaic animal with male and female structures, indicating that the ambiguous primary signal must have been transformed somewhere further down in the regulatory hierarchy into a clear signal for the sexual phenotype—female in some cells, male in other cells—of the individuum. A similar mosaic phenotype is produced by triploid intersexes (XX; AAA) with an ambiguous X:A ratio of 0.67.

The mosaicism described above might also apply to dosage compensation, with the female cells transcribing their X chromosomes at the typical low rate and the male cells at the high rate. Alternatively, all cells of a mosaic animal, male and female, might exhibit the same, intermediate transcription rate. In his discussion of sexually mosaic phenotypes displayed by certain XX; AA flies, Cline (1983; 1984) favors the first hypothesis. In sexual mosaics, the *Sxl*$^+$ gene is thought to be turned on in some cells, off in others, as a probabilistic response to an ambiguous signal. This possibility may also apply to triploid intersexes (discussed by Baker and Belote, 1983). According to this view, sex and dosage compensation, linked and mediated by *Sxl* being "on" or "off," would be strictly correlated, not only in normal males and females, but also in sexual mosaics. The alternative possibility is that an ambiguous X:A ratio leads to an intermediate state of activity of *Sxl* in all cells, which would trigger a uniform intermediate rate of transcription in male and female cells of a sexually mosaic animal.

The two possibilities—*Sxl*$^+$ "on" in some cells, "off" in others, and *Sxl*$^+$ active to intermediate levels in all cells—will now be compared with the results (Figure 8). Genotypes E and F (Figure 7b) have an identical X:A ratio, which is the signal that regulates *Sxl*; they only differ in having two or one *Sxl*$^+$ genes. Therefore, if the intermediate X:A ratio were to cause an activation of *Sxl* with a certain probability, cells should be found that failed to activate *Sxl*$^+$. The probability for this to happen may be lower in genotype E with two *Sxl*$^+$ genes than in genotype F where one of the alleles, *Sxl*^{f}, is defective by mutation. Cline (1984) proposed that an active *Sxl*, even when mutant, might be able to transactivate another allelic gene. But since an active gene is a prerequisite for transactivation, this phenomenon, if it exists, would not affect the frequency of cells with no active *Sxl*$^+$ genes in genotype E and we would expect to find male tissue in genotype E as well as in genotype F. This is not what we observed: no male clones were found in genotype E, whereas genotype F differentiated many male structures.

The model that assumes a probabilistic activation of *Sxl* can hardly explain why, as a response to the same intermediate X:A ratio, all cells of genotype E activate at least one *Sxl*$^+$ gene, whereas many cells of genotype F do not. The second model, which we favor, states that the intermediate X:A ratio activates all *Sxl*$^+$ genes in genotype E and F to an intermediate level of expression. Genotype F has levels of *Sxl*$^+$ product that are at the threshold for the sexual pathway so that some cells differentiate male, others female structures. Genotype E has twice as much *Sxl*$^+$ product as genotype F, which dictates the female pathway to every cell.

A similar argument applies to genotypes H and J (Fig-

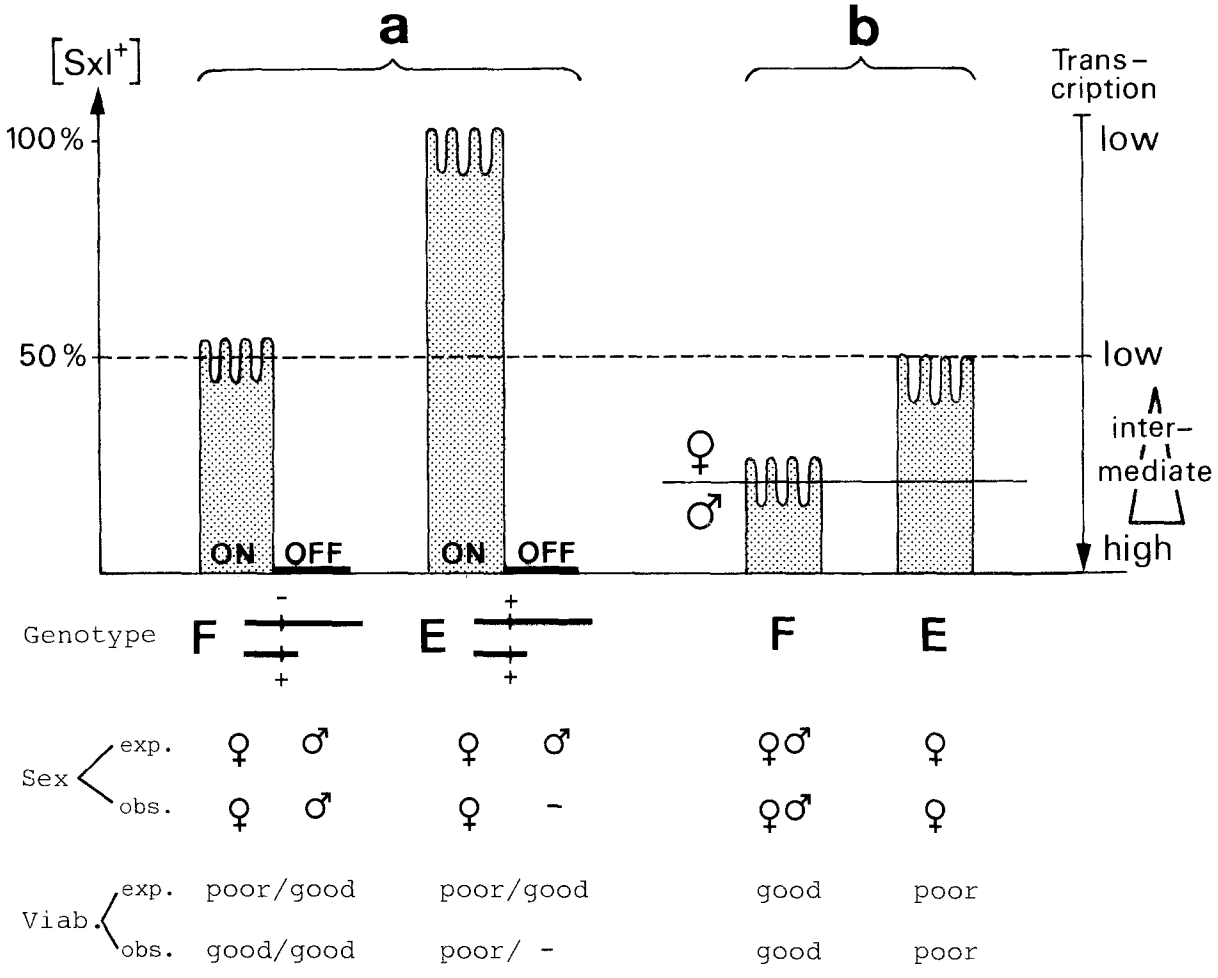


Figure 8. Comparison of the Two Views about the Effects of Ambiguous X:A Ratios on the Activity of *Sxl*+

(a) *Sxl*+ can be ON or OFF as a probabilistic response of cells so that the gene is active in some cells and inactive in other cells of an individuum. (b) *Sxl*+ assumes an intermediate level of activity in all cells.

The height of the shaded columns corresponds to the amount of *Sxl*+ product in a given cell (left ordinate, in % of a normal female). A high level of *Sxl*+ product implements a low rate of transcription, as indicated on the right ordinate. The dashed horizontal line indicates the minimum level of *Sxl*+ product needed to implement the low rate of transcription typical for normal females. The solid horizontal line represents the threshold level for the sexual response, female (♀) for higher levels, male (♂) for lower levels of *Sxl*+ product.

The figure shows the expected (*exp.*) and observed (*obs.*) consequences on sexual phenotype (*Sex*) and viability (*Viab.*). The two aneuploid genotypes, F and E, have the same intermediate X:A ratio, but F has only one *Sxl*+ gene (−/+), whereas E has two (+/+) (see Figure 7b). No conflict exists between expectation and observation for model (b), whereas model (a) suffers from discrepancies.

ure 7). Both lack one dose of region 3F3 to 4B1, which was shown to play a crucial role in the activation of *Sxl*; they only differ in having one or two *Sxl*+ genes. If the single *Sxl*+ gene in genotype H were set "on" or "off" as a random response to the presence of a single dose of region 3F3 to 4B1, then the two *Sxl*+ genes of genotype J should remain inactive in some cells, and hence male structures should also occur in genotype J. Flies of this genotype, however, are normal females.

The two models also make different predictions concerning dosage compensation. Contrary to the sexual phenotype, however, the transcription rate of the X chromosomes cannot be directly assessed in differentiated adult cells. But we know that in a mosaic animal, clones of cells heterozygous for a deficiency grow more slowly than normal cells or cells with a duplication; in extreme cases, cells carrying a deficiency may not be viable or may disappear due to cell competition, a process which can eliminate clones in all body regions except in the abdomen (Morata and Ripoll, 1975; Ripoll, 1980; Simpson, 1981; Simpson and Morata, 1981). We therefore expect that our aneuploid cells will grow better when their X chromosomes are transcribed at a high rate than when they are transcribed at a low rate. We have already seen that an aneuploid genotype survives better with an inactive *Sxl* gene which allows a high rate of transcription (Figure 7a). When an aneuploid genotype, such as A, transcribes its X-chromosomal genes at a low rate, it in essence corresponds to a female with a large deficiency; when it has a higher rate of transcription, such as genotype B, it is more like a male with a duplication. It has been generally observed that duplications are much better tolerated than deficiencies. Therefore, the relative growth capacities of clones may be used to measure the transcriptional activity of their X chromosomes.

Let us again compare genotypes E and F (Figure 7b).

If the intermediate X:A ratio were to cause random activation of *Sxl*$^+$ in some cells but not in others, the level of *Sxl*$^+$ product, in those cells in which *Sxl*$^+$ is "on," would be 50% of a normal female in genotype F, and 100% in genotype E. We know that a level of 50% is sufficient to implement the low level of X-chromosomal transcription typical for females (genotype G, Figure 7b; Lucchesi and Skripsky, 1981). Therefore, those cells in which *Sxl*$^+$ is "on" should have the same low rate of transcription in both genotypes, E and F, and hence should display the same viability. This is, however, not the case: female tissue developed poorly in genotype E (Figure 3e), whereas it developed normally in genotype F. Thus, only the female tissue of genotype E behaves as if the cells carried a deficiency, which is indicative of a low rate of transcription. Furthermore, male and female tissue coexisting in the same clone do not show any difference in viability (Figure 3f). These results are more easily explained if we assume that genotype F produces a low level of *Sxl*$^+$ product that allows a relatively high rate of transcription, whereas genotype E produces twice as much *Sxl*$^+$ product which implements a lower rate of transcription.

Taken together, we interpret our results to indicate that *Sxl*$^+$ can assume different levels of activity. This conclusion was also reached in a theoretical paper by Gadagkar et al. (1982), who proposed a model for the activation of *Sxl* by different X:A ratios. Lucchesi and Skripsky (1981) have shown that *Sxl*^{f}/+ heterozygous females with presumably 50% of *Sxl*$^+$ product display a normal female rate of transcription. We now want to propose that below this 50%, the transcriptional activity of the X chromosomes is inversely proportional to the level of *Sxl*$^+$ product, leading to intermediate rates of transcription (Figure 8). Intermediate rates of transcription have in fact been measured in larvae that were aneuploid for parts of the X chromosome (Maroni and Lucchesi, 1980). As for sex determination, we must assume that a threshold level of *Sxl*$^+$ product exists to which sex controlling genes farther down in the regulatory pathway, such as transformer (*tra*) or double sex (*dsx*), react with a clear alternative activity that implements either the male or female pathway.

This view can also account for the sexual mosaicism of triploid intersexes (XX;AAA). At the same time, it explains why earlier work with triploid intersexes led to the conclusion that female determining factors were more or less evenly distributed over the entire X chromosome. As we have shown, intermediate X:A ratios or one dose of region 3F3 to 4B1 can activate *Sxl* to intermediate levels, and we believe that this is also the case in XX;AAA animals. Since these flies are sexually mosaic, the level of *Sxl*$^+$ product must be around the threshold value for sex determination. In this situation, even a slight change in the level of *Sxl*$^+$ product will be critical. Thus, duplications and deficiencies of X chromosome material, even when these harbor only minor female determining factors, may already result in a noticeable shift in the sexual phenotype, as observed by Dobzhansky and Schultz (1934). Such minor sex determining components may in fact be present on several regions of the X chromosome.

We are still ignorant about the mechanism by which the primary genetic signal, the X:A ratio, operates. What is the contribution of the X chromosome, of the autosomes? How is this mysterious ratio assessed by *Sxl*? A model proposed recently by Chandra (1985) suggests that an autosomal factor present in both sexes in equal, but limited, amounts and that acts as a repressor for *Sxl*, is titrated against a number of binding sites located on the X chromosome. Since a female has twice as many binding sites as a male, all repressor molecules are bound so that *Sxl* is open for transcription, whereas in males with only half the number of binding sites *Sxl* is repressed. As a first attempt to understand the role of the X chromosome, we analyzed the effects of X-chromosomal fragments and showed that this chromosome is differentiated with respect to location, strength, and function of factors that are involved in the control of sex determination and dosage compensation.

Experimental Procedures

Production of Flies with Intermediate X:A Ratios

As a rule, flies with two sets of autosomes and an X:A ratio clearly between 0.5 and 1.0 are lethal; but the aneuploid genotypes can survive in cell clones of mosaic individuals, where their sexual phenotype can be analyzed. Therefore, we constructed females with two X chromosomes and a duplication of known size. Such hyperploid animals exhibit normal viability, but reduced fertility. One of the X chromosomes was the unstable ring chromosome, *In(1)w*^{vC} (Hinton, 1955; Hall et al., 1976), which is frequently lost in some of the early cleavage nuclei, giving rise to mosaic flies (Figure 1a and b). Different X–Y translocations, *T(X;Y)*, were used to obtain duplications for parts of the X chromosome. All chromosomes and fragments were marked in such a way that their presence or absence could be detected.

The following translocation stocks were used; their breakpoints on the X are given in parentheses: *T(X;Y)B29** (4C); *T(X;Y)B36** (5C); *T(X;Y)149* (6E); *T(X;Y)102* (7D); *T(X;Y)J8** (8C); *T(X;Y)B26** (9C); *T(X;Y)B44** (11A); *T(X;Y)B39** (11D); *T(X;Y)B24** (12E); *T(X;Y)B28** (13F); *T(X;Y)B35** (15B).

Unless specified, the chromosomes and mutations are described in Lindsley and Grell (1968). The translocations with an asterisk are described by Stewart and Merriam (1973). These authors give the breakpoint for *T(X;Y)B29* in 3E. Our own cytological examination, however, placed it in 4C (see Figure 6).

Once region 1A to 7D was found to promote female differentiation when present in two doses, this region was analyzed in more detail. We constructed females with the unstable ring X chromosome, *In(1)w*^{vC}, the duplication 1 to 7D, and an X chromosome that carried one of the following deficiencies in the duplicated area, which in effect reduces the size of the duplication (Figure 1c): *Df(1)svr* (1A1; 1B10–13); *Df(1)A94** (1E5^a; 2B15); *Df(1)w*^{vco} (2C1–2^a; 3C5–6^a); *Df(1)64c18** (2E1–2; 3C2); *Df(1)N*$^{164-105}$ (3C6–7; 3D2–3); *Df(1)dm*^{756}* (3C11; 3E4); *Df(1)A113** (3D6/3E1; 4F5^a); *Df(1)HC244** (3E8; 4F11–12); *Df(1)HF366** (3E8; 5A7); *T(1;2)rb*$^{+71g}$* (3F3 to 5E8; 23A15); *Df(1)RC40** (4B1; 4F1); *Df(1)C149** (5A8–9; 5C5–6); *Df(1)N73** (5C2; 5D5–6); *Df(1)HA32** (6E4–5; 7A6). The breakpoints are given in parentheses. The deficiencies with an asterisk were isolated by Dr. G. Lefevre (Craymer and Roy, 1980); (^{a}), breakpoints according to Dr. G. Lefevre. In all these chromosomes, the mutation *f*^{36a} was introduced as a cell marker.

In a further experiment we used the chromosome *T(1;2)w*^{64b13}, which is a second chromosome that carries an insertion for an X chromosomal segment (3C2 to 5A1–2 inserted in 26D; Craymer and Roy, 1980). In the text we refer to this chromosome as *Dp(3C2 to 5A1)*.

Scoring of Sexually Dimorphic Structures

The flies were raised at 21°C on standard Drosophila medium (cornmeal, agar, sugar, yeast, and Nipagin). The flies to be analyzed were macerated in hot 10% NaOH and mounted in Faure's solution for inspection under a compound microscope. The sex of the clones was assessed in sexually dimorphic areas, i.e. the sex comb region of the

foreleg, the external terminalia (genitalia and analia), the area of abdominal tergites 5 and 6 which is only pigmented in males, and sternite 7 and tergite 7, two structures that are only present in females. Missing sternites 7 and tergites 7 were scored as male when associated with a male tergite 6 (for an illustration of sexually dimorphic structures see Figure 3).

The sexual phenotype of a clone was sometimes ambiguous, mainly when the clone was small. These cases were not considered in the analysis. They mainly concerned small clones in the anal plates and on tergites 5 and 6. Similarly, bristles on sternites 6, usually a female characteristic, proved to be an unreliable female marker since sternites 6 with bristles have also been observed in males (Cline, 1979; 1983; Baker and Ridge, 1980).

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