Physiol. (London) **334**, 33 (1983); R. G. Morris, E. Anderson, G. S. Lynch, M. Baudry, *Nature (London)* **319**, 774 (1986).
33. R. J. Ferrante *et al.*, *Science* **230**, 561 (1985).
34. R. P. Simon, J. H. Swan, T. Griffiths, B. S. Meldrum, *ibid.* **226**, 850 (1984); T. Wieloch, *ibid.* **230**, 681 (1985).
35. M. J. Croucher, J. F. Collins, B. S. Meldrum, *ibid.* **216**, 899 (1982).

36. We thank M. Yokoyama for helpful discussion, V. Viseskul for assistance with cell cultures, and F. Smith for assistance with manuscript preparation. Supported by NIH grant NS21628, a grant from the Wills Foundation, NIH training grant NS07280 (to S.P.), and a Hartford Foundation fellowship (to D.W.C.).

16 January 1987; accepted 9 March 1987

A Chicken Transferrin Gene in Transgenic Mice Escapes X-Chromosome Inactivation

MICHAEL A. GOLDMAN, KAREN R. STOKES, REJEAN L. IDZERDA, G. STANLEY MCKNIGHT, ROBERT E. HAMMER,* RALPH L. BRINSTER, STANLEY M. GARTLER

Mammalian X-chromosome inactivation involves a coordinate shutting down of physically linked genes. Several proposed models require the presence of specific sequences near genes to permit the spread of inactivation into these regions. If such models are correct, one might predict that heterologous genes transferred onto the X chromosome might lack the appropriate signal sequences and therefore escape inactivation. To determine whether a foreign gene inserted into the X chromosome is subject to inactivation, transgenic mice harboring 11 copies of the complete, 17-kilobase chicken transferrin gene on the X chromosome were used. Male mice hemizygous for this insert were bred with females bearing Searle's translocation, an X-chromosome rearrangement that is always active in heterozygous females (the unrearranged X chromosome is inactive). Female offspring bearing the Searle's translocation and the chicken transferrin gene had the same amount of chicken transferrin messenger RNA in liver as did transgenic male mice or transgenic female mice lacking the Searle's chromosome. This result shows that the inserted gene is not subject to X-chromosome inactivation and suggests that the inactivation process cannot spread over 187 kilobases of DNA in the absence of specific signal sequences required for inactivation.

X-CHROMOSOME INACTIVATION IN mammalian females represents a unique kind of gene regulation in two respects. (i) The active and inactive states of each gene are maintained in the same nucleus, in contrast to the typical tissue-specific genes in which both copies of a gene are either active or inactive. (ii) The phenomenon involves not a battery of dispersed genes but most of the genes of a chromosome. X-chromosome inactivation (XCI) probably is initiated at a single site on the chromosome (*1*). The inactive state then spreads along the chromosome, encompassing all but the limited X-Y pairing and recombination region. In X-autosome translocations, the inactive state may spread into the autosome, may fail to do so, or may "skip" proximal autosomal regions while spreading into more distal ones (*2*).

A key question in our understanding of the XCI process is whether specific regulatory DNA sequences are required to render certain chromosomal regions susceptible to XCI and other regions immune. We report here a study of XCI in transgenic mice having the complete chicken transferrin gene, including 2.2 kb of 5′ and 3.7 kb of 3′ flanking sequence, on the X chromosome. McKnight *et al.* described the preparation of these mice and showed that 11 copies of the chicken transferrin gene were located on the X chromosome and were preferentially expressed in liver (*3*). We used complementary DNA (cDNA) solution hybridization techniques (Table 1) to determine the relative expression of the chicken transferrin gene in hemizygous males and in heterozygous females. If the transferrin insert were subject to XCI, the livers of heterozygous females should be a mixture of cells having the transferrin gene on the active X and those having the gene on the inactive X, whereas all liver cells of a male would express the inserted gene. Thus heterozygous females should produce a lower overall quantity of message than the males. Although our data showed no significant difference in transferrin gene expression between males and females (Table 1), variation in our assay system did not permit us to exclude the hypothesis of a twofold difference in expression between the available males and females.

To definitively show whether the chicken transferrin gene on the mouse X chromosome was subject to XCI, we took advantage of the Searle's translocation (*4*), a reciprocal exchange between the X chromosome and chromosome 16. When heterozygous in females, both segments of the rearranged X in Searle's translocation are active, whereas the normal chromosome is nonrandomly inactivated in all cells (*5*). We could therefore produce female mice heterozygous for Searle's translocation with the transferrin gene on the normal X chromosome and expect that the chromosome bearing the chicken transferrin gene would be inactive.

We mated males hemizygous for the chicken transferrin gene to females lacking the transferrin insert but heterozygous for the Searle's translocation with the unrearranged X chromosome marked with the *Tabby* (*Ta*) gene. All female offspring should have the chicken transferrin gene on an otherwise normal X chromosome, and this was confirmed by hybridization of the transferrin gene probe to dot-blotted DNA samples prepared from tail (*6*). Females inheriting the normal X chromosome instead of the Searle's rearrangement were identified by the *Ta* heterozygous phenotype, whereas females containing the Searle's translocation would appear phenotypically normal (*7*). The phenotypically normal female mice were killed, and livers were assayed for chicken transferrin gene expression. All three Searle's females tested showed levels of transferrin gene expression that were not significantly different from levels observed in transgenic males, demonstrating that the chicken transferrin gene insert was not inactivated when on the inactive X chromosome (Table 1). We cannot rule out the possibility that the level of expression of the transferrin gene insert on the inactive X is somewhat lower than that on the active X, or that some of the 11 copies of the gene are active while others are not. However, it is clear that complete inactivation does not occur.

In this report, we have demonstrated that 11 copies of the complete chicken transferrin gene, introduced onto the X chromosome of transgenic mice, escaped complete XCI. A gene inserted into the X chromosome might escape XCI because (i) it is located in the limited X-Y pairing and recombination region of the X chromosome, which normally escapes XCI, (ii) the insert-

M. A. Goldman, K. R. Stokes, S. M. Gartler, Departments of Medicine and Genetics and Center for Inherited Diseases, University of Washington, SK-50, Seattle, WA 98195.
R. L. Idzerda and G. S. McKnight, Department of Pharmacology, University of Washington, Seattle, WA 98195.
R. E. Hammer and R. L. Brinster, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104.

*Present address: Howard Hughes Medical Institute, University of Texas Southwestern Medical School, Dallas, TX 75235.

Table 1. Chicken transferrin gene expression in heterozygous female and hemizygous male mice and in female mice heterozygous for the chicken transferrin gene and Searle's translocation.

Transferrin gene expression* (pg/μg)					
Females		Males		Searle's heterozygotes	
$\bar{x}$ (SD)	*n*	$\bar{x}$ (SD)	*n*	$\bar{x}$ (SD)	*n*
Experiment 1					
4.2(1.5)	4	6.5(1.6)	4	8.1	1
Experiment 2					
7.1(2.7)	2	6.5(1.1)	2	4.3(0.7)	2
Experiments 1 and 2					
5.2(2.2)	6	6.5(1.4)	6	5.6(2.2)	3

*The chicken transferrin gene expression assay was carried out by solution hybridization of a ^{3}H-labeled chicken transferrin cDNA to total nucleic acid prepared from mouse liver, as described (*23*). Each hybridization was carried out at three different nucleic acid concentrations. A standard curve was made simultaneously from samples containing known quantities of chicken oviduct messenger RNA. Values are expressed as picograms of chicken transferrin RNA per microgram of total nucleic acids in liver tissue, plus or minus standard deviation. A value of approximately 5.5 pg/μg is equivalent to about 66 message molecules per cell and is in agreement with previous reports of message level in this transgenic mouse line (*3*). Nonliver tissues gave values approximately 1/10 those of liver (*3*). Control mouse livers lacking the chicken transferrin gene showed negligible hybridization to the probe.

ed gene or region lacks specific signal sequences that are required for XCI, or (iii) the insert has some sequence composition that renders it resistant to XCI.

It does not appear that the transferrin gene insert is in the pairing and recombination region of the X chromosome, because we have observed no recombination between the X and Y chromosomes involving this locus, among more than 130 informative progeny tested. In contrast to this insert, the *Sts* locus, which is the only known gene on the mouse X chromosome that normally escapes XCI, undergoes obligatory recombination between the X and Y chromosomes (*8*). Therefore the transferrin gene insert is not linked to the *Sts* locus and is likely to be outside the region of the chromosome that normally escapes inactivation. Experiments are in progress to map the insert precisely.

The *Sts* gene in the limited X-Y pairing region of the mouse X chromosome, as well as many autosomal genes in X-autosome translocations, is known to escape XCI (*1, 8*). Because few of these genes have been cloned or characterized, we have little insight into the question of whether specific sequences may be protecting these genes from XCI. The introduction of foreign or autosomal genes onto the X chromosome of mice through germline transformation, however, allows us to compare the abilities of known DNA sequences to undergo XCI. Two such cases are now available—that reported here and the mouse autosomal α-fetoprotein (AFP) gene inserted onto the X chromosome (*9*).

Krumlauf *et al.* studied the behavior of a mouse AFP minigene on the X chromosome of transgenic mice (*9*). Although the gene is normally autosomal, it was correctly inactivated in fetal liver tissue when present on the inactive X chromosome. This finding suggests that there are no sequences unique to the X chromosome that are required in the 5′ control regions or in the coding sequence of a gene in order for XCI to occur. These results are in agreement with previous reports of the spreading of XCI into translocated autosomal genes (*10*).

Three important differences between our experimental system and that of Krumlauf *et al.* (*9*) may shed light on the contrasting results. (i) They used a minigene construct, while we used an intact gene. (ii) Total insert size was 187 kb in the transferrin transgenic mice, but less than 45 kb in the AFP mice. (iii) The transferrin gene was cloned from chicken, an organism in which XCI does not occur, while the AFP gene was from a mouse. The difference in our results might be due to the nature of the inserted sequences, the amount of DNA inserted, the position of the insert on the chromosome, or a combination of these factors.

If specific sequences are required for XCI, one might expect to find sequences common to all X-linked genes, but lacking in genes that escape XCI. Sequence data are now available for the presumed control regions of several X-linked genes, including human *Pgk* (*11*), mouse and human *Hprt* (*12*), human *G6pd* (*13*), human *Factor VIII* (*14*), and *Factor IX* (*15*). Analysis of these sequences has failed to reveal any sequence common to the 5′ regions of X-linked genes. Therefore, any regulatory sequences common to all X-linked genes must lie outside the regions that have been sequenced or must lack extensive homology.

There is good evidence that XCI involves DNA methylation in CpG-rich islands near housekeeping genes (*1, 16*). We hypothesized that the chicken transferrin and mouse AFP genes might behave differently with respect to XCI because of a difference in their content of methylatable sequences. We analyzed the sequences of the 5′ regions of these genes and found that the chicken transferrin gene promoter (*17*) actually had a higher dG + dC content than the AFP promoter (*18*), and a marginally greater fraction of CpG dinucleotides, although neither region had CpG-rich islands. Thus, we have no evidence that the different behavior of these two genes results from a difference in number of methylatable sites, although we cannot rule out the possibility that critical CpG sites on the AFP gene are absent in the transferrin gene.

We hypothesize that the chicken transferrin gene insert escapes XCI because it occupies a region that is too large to undergo XCI in the absence of specific signal or recognition sequences ordinarily present at intervals along the inactivated region of the X chromosome. Chromosomal DNA in vertebrates is organized into discrete regions or domains of about 100 kb in length that are anchored to a protein matrix and form torsionally independent loops (*19*). These domains appear to be functional units in that the region as a whole may take on specific conformational characteristics when the region is capable of transcription (*20*). We propose that the functional unit of the spread and maintenance of XCI is such a chromatin domain. Because this domain is a unit considerably larger than the transcriptional unit itself, we predict that signal sequences required for XCI need occur only once per domain. Some autosomal genes are inactivated in X-autosome translocations because they occur in domains that have signal sequences. Others lack these sequences and therefore escape inactivation. The chicken transferrin gene, we believe, escapes inactivation because the 187-kb expanse in which it is found forms one or more independent domains that lack XCI signal sequences. The mouse AFP insert is subject to XCI either because it has such sequences or because it is included in a domain that has these sequences and therefore is already subject to XCI.

The nature of specific sequences required for XCI remains obscure. Cullen *et al.* (*21*) have suggested that CpG-rich islands (*22*) occurring at intervals of approximately 100 kb may be important in XCI. Competence for XCI may depend on the presence of such an island in each chromatin domain. The absence of such a CpG island in the 17-kb chicken transferrin gene insert is consistent with this notion.

REFERENCES AND NOTES

1. S. M. Gartler and A. D. Riggs, *Annu. Rev. Genet.* **17**, 155 (1983).
2. E. A. Keitges and C. G. Palmer, *Hum. Genet.* **72**, 231 (1986).
3. G. S. McKnight, R. E. Hammer, E. A. Kuenzel, R. L. Brinster, *Cell* **34**, 335 (1983); R. E. Hammer, R. L. Idzerda, R. L. Brinster, G. S. McKnight, *Mol. Cell. Biol.* **6**, 1010 (1986).
4. M. F. Lyon, A. G. Searle, C. E. Ford, S. Ohno, *Cytogenetics* **3**, 306 (1964).
5. S. Ohno and M. F. Lyon, *Chromosoma* **16**, 90 (1965). This apparent nonrandom inactivation may be achieved by random inactivation followed by selection, as cells that have inactivated the rearranged X are monosomic for a segment of chromosome 16. However, the matter is still controversial [N. Takagi, *Chromosoma* **81**, 439 (1980)].
6. R. D. Palmiter, H. Y. Chen, R. L. Brinster, *Cell* **29**, 701 (1982).
7. The *Tabby* gene is closely linked to the breakpoint in Searle's translocation and does not recombine significantly (*4*).

8. E. Keitges, M. Rivest, M. Siniscalco, S. M. Gartler, *Nature (London)* **315**, 226 (1985); E. Keitges and S. M. Gartler, *Am. J. Hum. Genet.* **39**, 470 (1986).
9. R. Krumlauf, V. M. Chapman, R. E. Hammer, R. Brinster, S. M. Tilghman, *Nature (London)* **319**, 224 (1986).
10. T. Mohandas. R. S. Sparkes, L. J. Shapiro, *Am. J. Hum. Genet.* **34**, 811 (1982).
11. J. Singer-Sam *et al.*, *Gene* **32**, 409 (1984).
12. D. W. Melton, C. McEwan, A. B. McKie, A. M. Reid, *Cell* **44**, 319 (1986); P. I. Patel *et al.*, *Mol. Cell. Biol.* **6**, 393 (1986).
13. G. Martini *et al.*, *EMBO J.* **5**, 1849 (1986).
14. J. Gitschier *et al.*, *Nature (London)* **312**, 326 (1984).
15. S. Yoshitake, B. G. Schach, D. C. Foster, E. W. Davie, K. Kurachi, *Biochemistry* **24**, 3736 (1985).
16. T. Mohandas, R. S. Sparkes, L. J. Shapiro, *Science* **211**, 393 (1981); J. A. M. Graves, *Exp. Cell Res.* **141**, 99 (1982); S. C. Lester, N. J. Korn, R. DeMars, *Somatic Cell Genet.* **8**, 265 (1982); P. H. Yen, P. Patel, A. C. Chinault, T. Mohandas, L. J. Shapiro, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1759 (1984); S. F. Wolf, D. J. Jolly, K. D. Lunnen, T. Friedmann, B. R. Migeon, *ibid.*, p. 2806; A. D. Riggs, J. Singer-Sam, D. H. Keith, *Progr. Clin. Biol. Res.* **198**, 211 (1985); D. H. Keith, J. Singer-Sam, A. D. Riggs, *Mol. Cell. Biol.* **6**, 4122 (1986); L. F. Lock, D. W. Melton, C. T. Caskey, G. R. Martin, *ibid.*, p. 914.
17. M. Cochet *et al.*, *Nature (London)* **282**, 567 (1979).
18. R. W. Scott and S. M. Tilghman, *Mol. Cell. Biol.* **3**, 1295 (1983).
19. J. A. Huberman and A. D. Riggs, *J. Mol. Biol.* **32**, 327 (1968); P. R. Cook and I. A. Brazell, *J. Cell Sci.* **19**, 261 (1975); C. Benyajati and A. Worcel, *Cell* **9**, 393 (1976); T. Igo-Kemenes and H. G. Zachau, *Cold Spring Harbor Symp. Quant. Biol.* **42**, 109 (1977); M. Buongiorno-Nardelli, G. Micheli, M. T. Carri, M. Marilley, *Nature (London)* **298**, 100 (1982); B. Vogelstein, B. D. Nelkin, D. M. Pardoll, S. I. Robinson, D. Small, in *ICN-UCLA Symposium on Gene Regulation*, B. W. O'Malley, Ed. (Academic Press, New York, 1982), pp. 105–119.
20. J. Stalder *et al.*, *Cell* **20**, 451 (1980); G. M. Lawson *et al.*, *J. Biol. Chem.* **257**, 1501 (1982); W. E. Stumph, M. Baez, W. G. Beattie, M.-J. Tsai, B. W. O'Malley, *Biochemistry* **22**, 306 (1983); M. A. Goldman, G. P. Holmquist, M. C. Gray, L. A. Caston, A. Nag, *Science* **224**, 686 (1984); M. Ryoji and A. Worcel, *Cell* **37**, 21 (1984); E. B. Kmiec and A. Worcel, *ibid.* **41**, 945 (1985); E. B. Kmiec, M. Ryoji, A. Worcel, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1305 (1986); E. B. Kmiec, F. Razvi, A. Worcel, *Cell* **45**, 209 (1986).
21. C. R. Cullen, P. Hubberman, D. C. Kaslow, B. R. Migeon, *EMBO J.* **5**, 2223 (1986).
22. A. P. Bird, *Nature (London)* **321**, 209 (1986).
23. G. S. McKnight, *Cell* **14**, 403 (1978).
24. Supported in part by NIH grants HD16659 to S.M.G., HD14412 to G.S.M., and HD17321 to R.L.B. S.M.G. is the recipient of an NIH research career award. We thank D. Adler, C. Disteche, K. Dyer, N. Ellis, G. Karpen, E. Keitges, and K. Swisshelm for comments on the manuscript.

17 December 1986; accepted 25 February 1987

Asymmetries in Mating Preferences Between Species: Female Swordtails Prefer Heterospecific Males

MICHAEL J. RYAN AND WILLIAM E. WAGNER, JR.

In male swordtails (*Xiphophorus nigrensis*) there are three size classes that derive from allelic variation at the pituitary locus on the Y chromosome. Progeny analysis and preference tests suggest that females prefer to mate with larger males. In the closely related *X. pygmaeus*, there is no allelic variation at this locus; this species consists of males similar in size only to smaller *X. nigrensis* males. In addition to being smaller than most *X. nigrensis* males, these *X. pygmaeus* males also lack both the swordtail and a major component of the courtship display common in most *X. nigrensis* males. Usually, female *X. pygmaeus* prefer to mate with heterospecific males rather than conspecifics, regardless of body size and the presence of a swordtail. However, the smallest *X. nigrensis* males lack the same courtship component as do the *X. pygmaeus* males, and in this comparison female *X. pygmaeus* show no preference. Although sexual selection, through its action on divergence of courtship displays, has been implicated as a factor leading to speciation, in this case sexual selection could lead to the congealing of gene pools between heterospecifics.

IN MANY SPECIES, MATING ATTEMPTS BY a male can be successful only with the cooperation of the female. Female choice can have important effects at two levels: it can result in mating with conspecifics instead of heterospecifics (interspecies discrimination), and it can enhance the mating success of some conspecific males relative to others (intraspecies discrimination). Mate choice usually is hierarchical; intraspecies discrimination, or sexual selection, acts within the constraints of interspecies discrimination, or species recognition. Furthermore, researchers have suggested that divergence of courtship signals under sexual selection can lead to speciation as individuals from different populations fail to recognize one another as conspecific (*1*). We report that females of two species of swordtail (*Xiphophorus*, Poeciliidae family) exhibit preference for mates and that in one species females prefer heterospecific males to their own conspecifics. This heterospecific preference results from lack of a courtship display component in conspecific males combined with similar preference by females of both species for full courtship display. These data suggest that sexual selection can not only have a diversifying effect, as suggested above (*1*), but can also override species recognition and potentially act as a congealing force between closely related species.

Many species of *Xiphophorus* are characterized by considerable variation in body size (*2*). Much of this variation is heritable [for example, greater than 90% for the Rio Choy, Mexico, population of *X. nigrensis* (*3*)], and it results from allelic variation at the pituitary (P) locus on the Y chromosome (*2*). In species with a greater number of alleles at the P locus, body size is distributed continuously, but in *X. nigrensis* from the Rio Choy, there are only three alternative alleles at the P locus, which results in three discrete body size classes (*2*). Swordtails have internal fertilization, females choose their mates, and less preferred males attempt to force copulation with females (*4, 5*). Paternity analysis of progeny from females collected in the field demonstrates a mating advantage for larger males in *X. nigrensis* (*6*). Laboratory tests reveal female mating preferences for these larger males that are consistent with the greater mating success of larger males in nature (*6*).

We wanted to determine if this preference for large males in *X. nigrensis* could be generalized to closely related species. If so, females of closely related species that did not have large males should prefer to mate with heterospecifics. This preference would constitute a unique example of mate choice overriding considerations of species recognition, and would demonstrate sexual selection that potentially gives rise to heterospecific preference. *Xiphophorus pygmaeus* and *X. nigrensis* are allopatric and closely related (*7*). In the former species, the P locus also influences male body size, but only the allele that results in smaller males is present (*3*). Therefore, we tested the hypothesis of heterospecific preference by giving female *X. pygmaeus* a choice between their own conspecific and a larger *X. nigrensis* male.

Xiphophorus pygmaeus contains gold and blue males; thus in these initial tests females were tested with either a gold [26 mm standard length (SL)] or a blue conspecific male (26 mm SL) against a larger heterospecific (37 mm SL). Eleven females were tested in four trials: twice with a blue conspecific-heterospecific pair, and twice with a gold conspecific-heterospecific pair. The testing apparatus was an aquarium (45 by 90 by 41 cm) that was divided into five equal sections. The sections at each end were separated from the three central sections by plexiglass. A male was placed in each of these end sections. The plexiglass partition ensured that females were exposed only to

Department of Zoology, University of Texas, Austin, TX 78712.