* Operated by Union Carbide Nuclear Company for the United States Atomic Energy Commission.

† The formal reporting of the gene *scurfy*, apparently the first sex-linked gene to be discovered in the mouse, was, of course, long overdue, and some explanation for the delay seems in order. Although *scurfy* behaved, in general, like a sex-linked gene, the early, and not too infrequent, occurrence of *scurfy* females, here reported, raised the possibility that we were dealing, instead, with a sex-limited gene which was occasionally expressed in females. By 1951, the ovarian transplantation results had, as shown in this paper, excluded this possibility. Proof of sex-linkage was considered adequate at this time, when tabulation of the offspring from transplanted *scurfy* ovaries showed all of 13 sons to be *scurfy*. At this same time, there were 7 adequately tested daughters, all of which transmitted *scurfy* to half of their male offspring. This led us to the conclusion that the exceptional *scurfy* females might be the result of an unexpectedly high frequency of non-disjunction in their mothers. The stock was turned over to an assistant to collect about twice as much material. Owing to the pressure of other work we did not look at the augmented data until some time later, when the keen interest of Dr. Curt Stern in our apparently high frequency of non-disjunction led us to tabulate the complete data. We then found out, for the first time, that some of the more recently obtained daughters of *scurfy* ovaries were *non*-transmitters of *scurfy*. It was our bad luck that of the first 7 adequately tested daughters *all* were transmitters of *scurfy*, and that we consequently had no inkling that the later data would contain an exciting new problem. When this problem turned up, hypotheses 4, 5, and 6, outlined in this paper, were proposed as possible explanations. Some time before this, an exceptional female had occurred in another stock maintained by one of us (LBR), in a cross of $X^{+Ta}/X^{26K+} \times X^{++}/Y$, and it was decided that exceptional sex-linked inheritance might be more easily analyzed in *Tabby* crosses, where ovarian transplantation is not necessary. The genetic and cytological results obtained with these crosses are reported in the accompanying paper.[1] They indicate that X/O is female, a finding which explains the exceptional females not only in the *Ta* crosses, but also in the old *scurfy* data. Thus, the remaining problem in the *scurfy* results has apparently been resolved, and the data are now at last presented.

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THE Y-CHROMOSOME AS THE BEARER OF MALE DETERMINING FACTORS IN THE MOUSE

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Introduction.—Russell, Russell, and Gower, in the accompanying paper,[1] report the occasional occurrences, over the course of several years, of an unexpected class of female progeny in matings of normal males with females heterozygous for the sex-linked mutation *scurfy*. These rare, unexpected, females phenotypically resemble the hemizygous males. Since the affected females die before reproducing, genetic analysis had to be attempted by means of ovarian transplantation. This was successful in several cases and the results, described in the companion paper,[1] ruled out a number of possible explanations for the exceptional *scurfy* females. Without further work, however, no decision was possible between the remaining hypotheses. The experiments to be described here have led to an unequivocal explanation of unexpected *X*-linked inheritance.

Since the time of the original experiments with *scurfy*, other sex-linked genes have become available. Their use permitted the design of crosses such that any exceptional females that occurred could be tested directly, without the tedium of ovarian transplant operations. The new sex-linked genes also allowed all three of the *X*-chromosomes involved in a cross to be marked. In addition to these improved means for genetic analysis, cytological analysis has recently become feasible in females by means of techniques developed for somatic tissues.

The hypotheses that remained to be tested following the experiments with *scurfy* were, briefly, (1) that the exceptional females were homozygous for the marker, due to non-disjunction in the mother, and (2) that the exceptional females were hemizygous due either to (*a*) an *X/O* chromosome constitution, or to (*b*) an X/X^{Df} chromosome constitution, with the deficiency involving the locus on the paternal *X*-chromosome, or to (*c*) an *X/Y* chromosome constitution which was phenotypically female as a result of loss of male determining factors on the *Y*. These hypotheses and the assumptions that have to be made in each case are outlined in greater detail in the accompanying paper.[1] While distinction between hypotheses 1 and 2 is possible by genetic analysis, decisions between the subhypotheses 2a, 2b, and 2c can be made only on a cytological basis. For the sake of brevity, the subsequent genetic discussion will refer only to hypotheses 1 and 2a, and it is understood that any conclusions applying to 2a (i.e., the *X/O* hypothesis) would, on the basis of the genetic evidence alone, also apply to 2b and 2c.

Non-disjunction in heterozygous females—either equational, or reductional preceded by crossing-over—could explain the retention of two mutant *X*-chromosomes within the functional egg cell. Following fertilization by a *Y*-sperm, this would result in exceptional females of constitution *X/X/Y*. On the other hand, results of breeding tests of the progeny from transplanted ovaries of *scurfy* females, discussed in the accompanying paper,[1] led to serious consideration of an *X/O* female constitution as an explanation for the unexpected females, although the existence of a fertile *X/O* female would mean that the mechanism of sex determination in the mouse is unlike that in *Drosophila*.[2] If *X/O* were female, one could look upon non-disjunction of the sex-chromosomes in the male as the basis for the origin of the unexpected class of females. Failure of the *XY* bivalent to disjoin at meiosis would result in sperm which carried neither an *X* nor a *Y* chromosome. Fertilization, by such a sperm, of an egg containing the mutant *X*-chromosome would then produce a female hemizygous for the marker and thus phenotypically resembling males bearing the marker.

To test the *X/X/Y* and *X/O* hypotheses, a series of crosses was performed in order to procure exceptional females which, in turn, could be subjected to a genetic analysis. The results of these genetic experiments combined with pertinent cytological observations are detailed below. They allow one to conclude that fertile females may have an *X/O* constitution. This would imply that in the mouse the *Y*-chromosome is male-determining.

Detection of New Exceptional Animals.—Three sex-linked mutants were used in this investigation. The mutant *scurfy*, *sf*, is described in the accompanying paper.[1] It is completely recessive in heterozygous females. Hemizygous males usually die around weaning age and always without reproducing. Another mutant arose at this Laboratory and appears by all phenotypic criteria to be a repeat of the *Tabby*

described by Falconer.[3] For the sake of simplicity, it will be called *Tabby*, *Ta*. This dominant mutant is viable and fertile in homozygous and hemizygous condition. The homozygous and hemizygous phenotypes appear to be identical and quite distinct from the heterozygous. The third mutant, a dominant called *26K*, also arose at this Laboratory. It is lethal in the hemizygous condition, and in heterozygous females produces a mottled coat color. Results of linkage tests with our *Ta* make it appear possible that *26K* is a repeat of *Mottled*, *Mo*,[4] which it resembles phenotypically.

Five different crosses were designed so that females of an unexpected phenotypic class could be detected, assuming that such exceptional females were either of the chromosomal constitution $X/X/Y$, with both of the X-chromosomes of maternal origin, or of constitution X/O, with the X being maternal. That is, the crosses were set up to screen for exceptions having a sex-chromosome constitution similar to that of the earlier observed *scurfy* females. In addition, 2 of the 5 crosses could also have detected X/O females in which the X was of paternal origin. Such a type was not found.

A brief summary of the exceptional females which were detected on the basis of their unexpected phenotypes may be found in Table 1. In cross 1, $+sf/++$ ♀ ♀ $\times$ $Ta+/Y$ ♂ ♂, in which all daughters were expected to show the phenotype characteristic of *Tabby* heterozygotes, two wild-type females were produced. On the basis of the $X/X/Y$ hypothesis, these females should be $++/++/Y$, or $+sf/++/Y$; on the basis of the X/O hypothesis, they must be $++/O$.

TABLE 1

SUMMARY OF THE EXCEPTIONAL FEMALES OBTAINED FROM VARIOUS CROSSES INVOLVING SEX-LINKED MARKERS

Cross	♀ ♀	♂ ♂	No. of Exceptions	Possible Genotype
1. $+sf/++ \times Ta+/Y$	154	152	2	$+/+/Y$* or $+/O$
2. $+26K/++ \times Ta+/Y$	121	88	0	$Ta/Ta/Y$ or Ta/O
3. $Ta/+ \times +/Y$	503	468	2	$Ta/Ta/Y$ or Ta/O
4. $+sf/Ta+ \times ++/Y$	125	132	2	$Ta/Ta/Y$ or Ta/O
5. $+26K/Ta+ \times ++/Y$	276	164	2	$Ta/Ta/Y$ or Ta/O

* May also be $+/sf/Y$.

Crosses 3, 4, 5 all produced females of unexpected phenotype. In this case the phenotype was homozygous or hemizygous *Tabby*. According to the two main hypotheses considered, such females should be genetically $Ta/Ta/Y$ or Ta/O, respectively.

Genetic Tests of Exceptional Animals.—In genetic tests designed to distinguish between the hypotheses, the exceptionally *Tabby* females were crossed with normal males, and the exceptional wild-type females were crossed with *Tabby* males. The expectations based on the assumption that the exceptional females are of the X/O constitution are easy to determine and will be considered first.

If the wild-type females obtained from cross 1 are X/O, their genotype must be $+/O$. Therefore, $+/O$ ♀ ♀ $\times$ Ta/Y ♂ ♂ should yield females which are $Ta/+$. with a phenotype typical for that of *Tabby* heterozygotes, and Ta/O, with a phenotype like that normally found in homozygous *Tabby* females or hemizygous males. All the males should be $+/Y$, wild-type; it may be assumed that the O/Y condition is lethal (see below). For the *Tabby* exceptions found in cross

3, 4, and 5, the mating plan Ta/O ♀ ♀ × $+/Y$ ♂ ♂ would yield heterozygous *Tabby* females, and $+/O$ females with a wild phenotype. In this case all of the males would be Ta/Y. As can readily be seen from the upper portion of Table 2, these types of animals, expected on the X/O hypothesis, are the only ones that have been found to date in the progeny of the primary exceptions.

TABLE 2

OFFSPRING OBTAINED FROM MATING EXCEPTIONAL FEMALES (PRESUMED X/O CONSTITUTION)

Origin	Mating Plan	♀ ♀				♂ ♂		
		Ta/+	*+/O*	*Ta/O*	N. C.†	*Ta/Y*	*+/Y*	N. C.†
Cross 4	♀ 47* *Ta/O* × *+/Y*	2	..	..	1	..	..	1
Cross 4	♀ 59 *Ta/O* × *+/Y*	1	1	..	1	1	..	2
Cross 5	♀ 111* *Ta/O* × *+/Y*	3	..	..	1	5	..	4
Cross 3	♀ 893 *Ta/O* × *+/Y*	7	7	..	..	12	..	1
Cross 3	♀ 894 *Ta/O* × *+/Y*	2	1	..	..	..	..	..
Cross 1	♀ 187 *+/O* × *Ta/Y*	7	..	3	..	..	5	..
♀ 893	♀ 1144 *+/O* × *Ta/Y*	3	..	4	..	..	4	..
♀ 893	♀ 1146* *+/O* × *Ta/Y*	2	..	1	..	..	5	..
♀ 894	♀ 1174 *+/O* × *Ta/Y*	2	..	1	..	..	2	..
♀ 893	♀ 1355 *+/O* × *Ta/Y*	..	..	..	1	..	..	1
♀ 187	♀ 3 *Ta/O* × *+/Y*	2	..	..	..	4	..	..
Total		31		18	4		38	9

The upper portion of the table summarizes tests of the primary exceptions; the lower portion summarizes tests of presumed X/O daughters of the primary exceptions.
* Sacrificed for cytological observation.
† N. C. = died before classification as to phenotype was possible.

The expectations based on the assumption that the exceptional females are $X/X/Y$ will be briefly outlined for the *Tabby* exceptional females, which, according to the hypothesis, are $Ta/Ta/Y$. Such females would form gametes predominantly of types X^{Ta} and $X^{Ta}Y$ which, in the cross to X^{+}/Y males would produce X^{+}/X^{Ta} and $X^{+}/X^{Ta}/Y$ daughters and X^{Ta}/Y and $X^{Ta}/Y/Y$ sons (the latter possibly lethal). That is, no wild-type daughters are expected from the predominantly formed gametes, unless $X^{+}/X^{Ta}/Y$ is assumed to have a wild-type phenotype. Yet, as the upper portion of Table 2 shows, 9 wild-type daughters were produced from this cross. Gametes expected to be formed rarely by $X^{Ta}/X^{Ta}/Y$ females, namely, $X^{Ta}X^{Ta}$ and Y, would produce, in addition to two probably lethal types, daughters of the homozygous *Tabby* phenotype, $X^{Ta}/X^{Ta}/Y$, and wild-type sons, X^{+}/Y, neither of which was observed.

Thus, the progeny tests of the primary exceptions are in disagreement with the $X/X/Y$ hypothesis, but in complete agreement with the X/O hypothesis. Further confirmation of this hypothesis comes from the progeny of the presumed X/O daughters of the primary exceptions (lower portion of Table 2).

The test for the death of Y/O's cannot be as clear-cut as in the data of Russell *et al.*[1] because of the absence of data from parallel crosses which show the proportion of males when Y/O is not one of the progeny classes. However, when a reasonable adjustment is allowed for this factor, and correction made for the relative inviability of X/O daughters, the observed proportion of males is statistically consistent with the conclusion, already established by the *scurfy* data,[1] that Y/O animals die prenatally.

To summarize the genetic data, it may be stated that there is compelling evidence that the X/O hypothesis is to be preferred to the $X/X/Y$ hypothesis. In a strict

sense, as noted in the Introduction, this genetic evidence indicates merely that the exceptional females are hemizygous for the marker gene. They could carry a deficiency for the wild-type allele. Or their chromosomal constitution could be X/Y, phenotypically female as a result of loss of male determining factors on the Y. The subsequent cytological analysis will distinguish between these alternative possibilities for a hemizygous female genotype.

Cytological Tests of Exceptional Animals.—Chromosome counts performed on the exceptional females can, of course, settle the question. If these females are X/Y or X^{Df}/X, the chromosome count should be 40; if they are X/O, only 39 chromosomes should be found. If, contrary to genetic indications, they are $X/X/Y$, they should have 41 chromosomes.

In order to obtain preparations suitable for chromosome counts, the following technique was used. Mice were injected with 0.2 ml of 0.2 per cent colchicine. One and a half hours later the animals were killed, the femurs removed, and the marrow was washed out of the bone with a 20 per cent Tyrode solution and allowed to remain in this hypotonic solution for 11 min. This was followed by gentle centrifugation for 4 min. The supernatant was poured off, and the moist pellet was fixed by the addition of acetic-alcohol, a drop at a time. The cells were resuspended and a drop or two of the suspension was placed on a slide and allowed to dry until only a very thin film of fixative remained. A small drop of lactic-acetic orcein† (2 per cent orcein in equal parts of glacial acetic and 85 per cent lactic acid) was added and a coverslip immediately placed over the drop. The cells were stained for at least 15 min (lactic-acetic orcein dries very slowly) and then firmly squashed.

The technique described above was developed after several attempts to adapt the air-drying technique described by Rothfels and Siminovitch[5] had failed. Air-drying was abandoned because it was found that marrow cells were not spread well enough for accurate chromosome counts.

In an early modification of the technique, a drop of aceto-orcein was added to the cell suspension on the slide just before the slide was completely dry. A coverslip was placed over the stain and the preparation was immediately squashed. Better spreading was sometimes obtained, but the slides were of unpredictable quality and the brittleness of the fixed tissue often resulted in a great deal of cellular fragmentation. Because it had been observed by the senior author that lactic-acetic orcein seemed to have a softening effect upon the testicular tissue of *Drosophila*, it was hoped that this stain would counteract the excessive brittleness caused by the fixative. Consequently, the lactic-acetic orcein was substituted for aceto-orcein and slides of good quality were then produced. In fact, good preparations were made from material that had been in fixative for as long as four days; longer periods of fixation have not been tested.

The chromosome counts made from the bone marrow cells of normal and presumed X/O females are listed in Table 3. Rows 1 and 2 show the results obtained from counting cells of X/X animals. These counts were made in order to test the reliability of the technique. The first animal was genetically $+/+$ or $sf/+$, the other was $Ta/+$. Rows 3 and 4 show the results obtained from a normal animal and a presumed X/O matched as closely as possible for all points of technique. The slides were coded, then mixed and counted by Dr. E. F. Oakberg. The X/X

animal was genetically +/+ and the *X/O* was ♀ 1146 of Table 2, a daughter of one of the exceptional females obtained from cross 3. The last two rows of Table 3 show the counts obtained by the senior author on two of the primary exceptions, ♀ 47 and ♀ 111 (see Table 2), presumed to be *Ta/O*.

TABLE 3

SUMMARY OF CHROMOSOME COUNTS PERFORMED ON NORMAL AND *X/O* FEMALES

Genotype	Chromosome Number								
	34	35	36	37	38	39	40	41	42
sf/+ or +/+	0	2	2	3	1	2	20	2	0
Ta/+	0	0	2	3	0	4	30	2	1
+/+	0	1	1	2	0	5	30	0	0
+/*O* ♀ 1146*	0	1	1	0	10	43	1	0	0
Ta/O ♀ 47	1	0	2	2	2	32	2	0	0
Ta/O ♀ 111	0	1	0	3	2	38	0	0	0

* Coded series.

The data of Table 3 present convincing evidence that the females which were presumed to be of the *X/O* constitution lack a chromosome, and the genetic data of Table 2 indicate that the missing element is an *X*-chromosome. Since both *X/X* and *X/O* constitutions are female, while *X/Y* is male, the conclusion can be drawn that the *Y*-chromosome is male determining in the mouse.

Discussion.—It is possible to use the data of Table 2 to estimate the frequency of matroclinous *X/O* females. Allowance must be made for the fact that in crosses 2 and 5 only half of the *X/O* females would survive, and in cross 3 only one type of *X/O* female would be detected. The expectations from cross 4 and 5 are complicated by recombination between the sex-linked mutants (approximately 44 per cent in cross 4, and 4 per cent in cross 5): *X/O*'s derived from one crossover class are undetectable in cross 4; while in cross 5, one crossover class could not be detected, and the other would not survive. Taking account of these various consideratons, it may be calculated on the basis of the data that approximately 1.2 per cent of all females are of the matroclinous *X/O* constitution. If this *X/O* constitution is the result of meiotic loss or non-disjunction of the sex chromosomes in the male, the frequency of sperm without an *X* or a *Y* is approximately 0.6 per cent. The *X/O* condition could also result from loss of the paternal sex chromosome during the first cleavage division of the zygote. To date, all *X/O* females obtained inherited their *X*-chromosome from the mother. Whether or not patroclinous *X/O* females also occur remains to be more fully tested. In the present experiments, they could only have been detected in crosses 1 and 2.

Since the results here reported indicate the *Y*-chromosome to be male determining in the mouse, a situation quite different from that found in *Drosophila*,[2] certain speculations as to the sex of an *X/X/Y* animal are possible. From the demonstration that both the *X/X* and *X/O* constitutions are female, one might deduce that all female determining factors are autosomal, a situation which is exactly the reverse of that found in *Drosophila*. If this were so, *X/X* females and *X/Y* males would each have a full set of female determiners, and the male determiners on the *Y* would have to be quite strong in order to shift development completely to maleness. Furthermore, with all female determiners on the autosomes, *X/X/Y* constitution would be male, inasmuch as *X/Y* and *X/X/Y* animals would the have equivalent sets of sex determiners. On the other hand, it might be specu-

lated that at least some female determiners are carried by the X-chromosome. If this is the case, they must be of such potency that a single dose, as in the X/O constitution, is sufficient to shift development completely to femaleness when a Y is not present. With at least some female determiners on the X, an $X/X/Y$ condition might be intersexual. With most, or all, female determiners on the X, an $X/X/Y$ condition could be intersexual or possibly even female.

The recent case, described by Jacobs and Strong,[6] of a human intersex with an apparent $X/X/Y$ constitution indicates that such a constitution in mammals may cause intersexual development, but additional evidence is necessary before such a conclusion can be definitely drawn. It seems likely that the male determining potency of the Y-chromosome, demonstrated in the mouse, may apply to other, perhaps all, mammals, but details concerning the distribution and strength of both male and female sex-determining factors in a given mammalian genome might differ from species to species.

The crosses listed in Table 1 were so constructed that an $X/X/Y$ constitution resulting from fertilization of a normal egg by an XY sperm could be detected if it resulted in a phenotypic male or male-like intersex. If such were the case, the various crosses would yield males, or near-males, with phenotypes not normally found in the male (namely: heterozygous *Tabby* in all 5 crosses; and *26K*, either alone, as in cross 5, or combined with *Ta* as in cross 2). No such animals have yet been found. If the $X/X/Y$ constitution was produced and survived in these experiments, it must have been externally a female.

Summary.—Genetic experiments utilizing sex-linked markers have led to the detection of unexpected phenotypic classes of females. When tested in additional crosses, these exceptional females behave in a manner consistent with the hypothesis that they are hemizygous for the X-chromosome. Chromosome counts performed on the bone marrow indicate that these exceptional females have 39 chromosomes, as compared to 40 found in the controls. Thus, the cytological observations indicate that the exceptional females lack a chromosome, and the genetic evidence indicates that the missing element is an X-chromosome. It is concluded that the exceptional females have an X/O constitution.

Since a fertile female can be of the X/X or X/O constitution, it follows that the Y-chromosome of the mouse is male determining. This may apply to other, perhaps all, mammals, including man.

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* Operated by Union Carbide Nuclear Company for the United States Atomic Energy Commission.

† The use of lactic-acetic orcein was suggested to this laboratory by Dr. Jack Schultz. It is used here primarily for studies of *Drosophila* salivary gland cytology.

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