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Sex Inversion as a Model for the Study of Sex Determination in Vertebrates [and Discussion]

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Sex inversion as a model for the study of sex determination in vertebrates

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As a consequence of genetic sex determination, the indifferent gonadal blastema normally becomes either a testis or an ovary. This applies to mammals and to the majority of non-mammalian vertebrates. With the exception of placental mammals, however, partial or complete sex inversion can be induced in one sex by sexual steroid hormones of the opposite sex during a sensitive period of gonadogenesis. There is evidence that also during normal gonadogenesis in these species, in the XY/XX mechanism of sex determination testicular differentiation is induced by androgens, and in the ZZ/ZW mechanism, ovarian differentiation by oestrogens. In either case, the hormones may act via serological H-Y antigen as a morphogenetic factor. In contrast, in placental mammals including man, primary gonadal differentiation is independent of sexual steroid hormones, and factors directing differential gonadal development have not yet been conclusively identified. However, various mutations at the chromosome or gene level, resulting respectively in sex inversion or intersexuality, have provided clues as to some genes involved and their possible nature. In this context also, serological H-Y antigen is discussed as a possible factor acting on primordial gonadal cells and inducing differential growth or morphogenesis or both. The data available at present allow a tentative outline of the genetics of sex determination in placental mammals.

Introduction

Sex determination becomes manifest phenotypically when the indifferent gonadal blastema differentiates into either testis or ovary. The mechanism of sex determination resulting in differential gonadal development must include multiple steps which are to a large extent unknown. However, this multistep process can be disturbed either by mutation or by experimental interference, resulting in anomalous gonad differentiation and thus in partial or complete sex inversion. Spontaneous occurrence and experimental induction of sex inversion can therefore provide some insights into the mechanism of sex determination.

I present some examples of sex inversion pointing to a possible role of serological H-Y antigen in the mechanism of sex determination. The evidence is still circumstantial, and the gaps in our knowledge must be filled by hypotheses. However, these hypotheses can be tested, and it is therefore to be expected that the relevant experiments will be done in the near future. In this context, I should like to point also to the stimulating considerations of Polani (1985).

The serological H-Y antigen referred to here is identical with the Sxs antigen of Wiberg (1987). The problem of the existence of several H-Y antigens is not addressed in this paper. I use the term 'serological H-Y antigen' rather than SDM (serologically detected male) antigen because I deal with non-mammalian vertebrates as well as with mammals, so that the sex-specific antigen may be a female antigen (e.g. in birds).

It is well established that in normal sexual development, serological H-Y antigen is characteristic of the heterogametic sex (Ohno 1979; Wachtel 1983). In the XY/XX mechanism of sex determination the male, and in the ZZ/ZW mechanism the female, types H-Y positive, whereas the respective homogametic sexes are H-Y negative by definition. This definition does not exclude the possibility that some residual H-Y activity also occurs in the homogametic sex, but there is at least a distinct quantitative sex difference.

With the exception of placental mammals, it has been shown in various species belonging to different taxonomic categories of vertebrates that gonadal differentiation of one sex can be influenced by the sexual steroid hormone of the opposite sex (Mittwoch 1973). Taking the chicken as an example, administration of oestradiol to the male embryo at the indifferent stage of the gonadal anlage results in the transient formation of an ovotestis. However, the complementary experiment is not successful: administration of testosterone to the female chicken embryo does not change gonadal sex, and an ovary is formed. In this species, the female is the heterogametic (ZW) and the male the homogametic (ZZ) sex. Although in the female oestrogens are produced before ovarian differentiation, testosterone in the male is found only after the testis has developed. From these findings it may be assumed that, during normal development also, ovarian differentiation depends on oestrogens whereas testicular differentiation is independent of sexual steroid hormones. Thus in the ZZ/ZW mechanism of sex determination, the heterogametic female is the induced sex whereas the homogametic male is the constitutive sex. As oestradiol serves as an inducer in this case, it is also able to sex-invert the indifferent gonadal anlage of the male, at least to some extent, and an ovotestis is formed. In contrast, testosterone is not an inducer of primary gonadal development, and therefore has no influence on the female gonadal anlage (Taber 1964).

Because of the postulated role of serological H-Y antigen in primary gonad differentiation (Wachtel *et al.* 1975; Ohno 1976), and based on the findings mentioned above, it could be predicted that oestradiol controls the expression of serological H-Y antigen. If so, the female chicken embryo should be serological H-Y antigen negative at the indifferent stage of the gonad and before oestrogens occur physiologically. Moreover, the male which normally types negative for serological H-Y antigen should become positive after experimental sex inversion by oestradiol. Both predictions have been shown to be correct. The early female chicken embryo is negative for serological H-Y, and becomes positive at the time when ovarian development starts (Ebensperger *et al.* 1988*a*), whereas males, sex-inverted by oestradiol, become positive for serological H-Y antigen in their gonads. This has been shown not only in the chicken *in vivo* (Müller *et al.* 1979*b*) and *in vitro* (Ebensperger *et al.* 1988*b*), but also in other species with a ZW mechanism, e.g. the quail (Müller *et al.* 1980; Zaborski *et al.* 1981) and *Xenopus* (Wachtel *et al.* 1980). For a synopsis see Zaborski (1985).

In the XY mechanism of sex determination, one would expect the situation to be complementary to that in the ZW mechanism. Here, the male is the heterogametic (XY) and the female the homogametic (XX) sex. It is well known that in some teleostean and amphibian species with male heterogamety, the female can be sex-inverted by testosterone. Using the labrid fish *Coris julis* as a model, a hermaphroditic protogynous species with females and primary and secondary males, Reinboth (1975) has shown that females can be changed into secondary males by injections of testosterone. Our studies on serological H-Y antigen revealed that the female is negative whereas both types of male are positive for this antigen. We wondered if the female became positive after experimental sex inversion, and this was indeed the case

(Reinboth *et al.* 1987). Thus in this model, testosterone not only induces testicular development, but also appears to control the expression of serological H-Y antigen.

If, at this point, some generalizations are allowed, I could put forward the following hypothesis which, however, only apply to non-eutherian vertebrates.

1. In the ZZ/ZW mechanism of sex determination, oestrogens are the inducers of the ovary, and in the XY/XX mechanism, androgens are the inducers of the testis.

2. Primary gonadal development in the heterogametic sex is induced, whereas in the homogametic sex it is constitutive.

3. The sexual steroid hormones characteristic of the heterogametic sex control serological H-Y antigen, which has a functional role in the organization of the heterogametic gonad.

On these assumptions, either oestrogens or androgens control serological H-Y expression, depending on the type of sex determination. In addition, one and the same factor, serological H-Y antigen, is involved either in ovarian or in testicular morphogenesis, again depending on the type of sex determination. From this it can be concluded that this antigen serves the function of a non-specific signal to which the target cells, i.e. the somatic cells of the gonadal blastema, react in an autonomous way. However, the type of sex determination defines whether these cells react to the presence of the antigen by differentiating in a male or female direction.

In placental mammals, the mechanism of sex determination does not employ sexual steroid hormones (Jost 1947). Primary gonadal differentiation must have become hormone independent during evolution, presumably as a consequence of placentation. However, serological H-Y antigen is present in the heterogametic male sex and absent in the homogametic female sex (at least by definition based on serological criteria). Serological H-Y antigen is found in the early mammalian embryo already (Krco & Goldberg 1976), and therefore should be present in the male at the time of differential gonadal development also, before androgens are produced. Androgen independence of serological H-Y antigen follows also from the finding that in the syndrome of testicular feminization in man (Koo *et al.* 1977) and mouse (Bennett *et al.* 1975), owing to androgen resistance, this antigen is present. Thus serological H-Y antigen is not controlled by androgens in the mammalian male. This does not exclude, however, that it is controlled by sex-determining genes, and there is experimental evidence that this is indeed the case (Wolf (1985), and see below).

I shall not summarize here the various arguments supporting the hypothesis that serological H-Y antigen has a role in primary testicular differentiation in mammals including man, because there is abundant literature on this subject (for review, see Ohno 1979; Wachtel 1983). Instead, I shall concentrate on a special case: the occurrence of sex reversal in the mouse, when the Y chromosome of feral mice of the subspecies *Mus musculus domesticus* is transferred into the background of the C57BL laboratory strain. I have referred earlier to some preliminary findings on serological H-Y antigen in these mice carrying a foreign Y chromosome (Wolf 1985), and Eicher & Washburn (1986) were right to complain that detailed data on this important question were not yet published. In the meantime, we have finished our studies on this subject, and I shall present them briefly here because they may contribute to the problem of serological H-Y antigen and testis differentiation. (For details, an article is in preparation on this subject matter.)

The animals were provided by Dr Winking of Lübeck who mated feral male mice from four different localities (southern Germany, Switzerland, northern Italy and Yugoslavia) with

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C57BL females, and who established four different lines by repeated back-crossing of hybrid males to C57BL females. From the second back-cross generation onwards a highly variable pattern of sexual differentiation occurred, and in general a quarter of all XY individuals developed as intersexes or females. With a few exceptions, XY females were sterile. I shall refer here to our findings on serological H-Y antigen only.

Serological H-Y antigen was determined by using two different assays: the Raji-cell cytotoxicity test (Fellous *et al.* 1978) and a urease-ELISA (Bradley *et al.* 1987). The results of both methods were essentially identical. In most animals, non-gonadal tissues (kidney, spleen, liver) were tested and gonadal sex was carefully analysed histologically. In some animals, gonads were used to absorb antisera raised against serological H-Y antigen, and here, gonadal sex was identified by morphological inspection. As can be seen in table 1, serological H-Y antigen status and gonadal differentiation were closely correlated. XY females with ovaries on both sides were negative for the antigen throughout, whereas true hermaphrodites with the simultaneous occurrence of testicular and ovarian tissue were positive, the titre being reduced compared with male controls in some cases.

TABLE 1. SEROLOGICAL H-Y ANTIGEN IN XY FEMALE AND HERMAPHRODITE MICE (C57BL-Y^{DOM})

no. individuals tested	gonads[a]	tissue studied[b]	H-Y antigen activity
6	O/O	gonads	not detectable
2	O/O	gonads	not detectable
		K	not detectable
15	O/O	S, K, Li, Lu, H	not detectable
3	O/T	gonads	intermediary
		Li	intermediary
12	O/T or O/OT	S, K, Li, Lu	intermediary or full positive
1	T/OT	Li	intermediary
1	T/T	Li	full positive

[a] O, ovary; T, testis; OT, ovotestis.
[b] S, Spleen; K, kidney; Li, liver; Lu, lung; H, heart.

Interestingly, similar findings were recently reported for the horse by Kent *et al.* (1988). In this species, an inherited trait is the occurrence of XY mares, showing a variable degree of sex-reversal. Some XY females are even fertile, others exhibit gonadal dysgenesis, and still others are virilized. Here again, the degree of sex inversion and the serological H-Y status were correlated; the nearly normal XY females and some females with gonadal dysgenesis were negative for the antigen whereas the more virilized animals were positive.

Whatever the genetic basis is for these two examples of sex inversion, the negative correlation between serological H-Y antigen and feminization is striking. In the general context that in mammals, males are positive for serological H-Y whereas females are not, these findings confirm the rule that, when testicular tissue is present in an individual, serological H-Y antigen is also present.

Up to this point, with respect to placental mammals, the following two hypotheses can be put forward.

1. Serological H-Y antigen expression has become independent of sexual steroid hormones, possibly in connection with the evolution of placentation, and may be under the direct control of sex-determining genes.

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2. Serological H-Y antigen has kept its functional role in the primary differentiation of the heterogametic gonad, and this is the reason for the positive correlation between testicular tissue and this antigen.

As to the biological function of serological H-Y antigen, the evidence is still circumstantial, and we must await its biochemical characterization which is under way, before conclusive experiments can be done. It is to be said in this connection that the biological role of this antigen cannot be confined to primary testicular morphogenesis (if it has this function). It has been shown that the concentration of serological H-Y antigen in the mammalian testis increases towards puberty (Müller *et al.* 1978*b*), and also that early male germ cells are negative for this antigen and become positive when they differentiate (Zenzes *et al.* 1978; Bradley & Heslop 1988). Under this view it is tempting to speculate that serological H-Y antigen may interact also with testis maturation or spermatogenesis or both. In this connection, the postulate by Burgoyne (1987) that H-Y antigen as detected by cytotoxic T-cells (CML assay) plays a role in spermatogenesis is of interest. However, the molecular and functional relationships between the serological H-Y antigen and that detected by T-cells remains to be clarified first.

Sex inversion phenomena have also contributed to a delineation of the genetics of sex determination and serological H-Y antigen. I do not intend to review all the various data contributing to our present picture, but should rather like to discuss briefly my original conception (Wolf 1978) in the light of some recent findings pertaining to this problem.

My model postulates that the structural gene for serological H-Y antigen is autosomal. This is for various reasons. The Y chromosome can be excluded because serological H-Y antigen is found in individuals lacking this chromosome, e.g. XX true hermaphrodites (see, for example, Waibel *et al.* 1987) and possibly some XX males. A phylogenetic argument is that, in non-mammalian vertebrates, serological H-Y antigen can be induced in the homogametic sex, thus the structural gene is present in both sexes, whereas the Y chromosome is not. The X chromosome is not a good candidate because in polysomies of the X in the presence of the Y, titre of serological H-Y antigen decreases with an increasing number of X chromosomes (Fraccaro *et al.* 1982). If the structural gene were X-linked, H-Y activity should either show a positive gene–dose relation or remain on the same level, depending on whether or not the gene undergoes inactivation.

However, an autosomal localization is to be favoured not only by exclusion because there are various examples in mammals including man of autosomal mutations causing sex reversal. A particularly interesting case is the occurrence of XY females with ovaries in campomelic dysplasia, a condition with a probably autosomal recessive mode of inheritance. These sex-inverted patients lack serological H-Y antigen (Bricarelli *et al.* 1981), and the simultaneous occurrence, to a variable degree, of multiple malformations in these patients can be interpreted as the consequence of a submicroscopic chromosomal deletion including the structural gene for this antigen and one or several neighbouring genes. Autosomally caused sex-inversion is also known in the mouse (Eicher & Washburn 1986), the goat (Wachtel *et al.* 1978), and some other species. The recent reports by Lau *et al.* (1987, 1988), assigning a gene for a male-enhanced antigen which is a candidate gene for serological H-Y antigen to chromosome 6 of man and to chromosome 17 of the mouse, can be taken as further support for an autosomal localization of the structural gene for this antigen in mammals.

Obviously, the Y chromosome is involved in male determination, and a testis-determining factor (TDF) has for long been postulated to be encoded by this chromosome. The TDF gene

(*TDF*) has been mapped on the distal short arm of the human Y chromosome, and recently a DNA sequence which may code for a regulatory protein considered to be TDF has been cloned (Page *et al.* 1987). A gene exerting a positive control function on serological H-Y antigen has also been assigned to the Y-chromosome short arm. The assignment was based on deletion mapping on the one hand (Rosenfeld *et al.* 1979), and on the presence of this antigen in XX males on the other hand. XX males have been shown to carry various sized segments of the Y chromosome on one of their X chromosomes, but they all include the *TDF* region (Vergnaud *et al.* 1986, Affara *et al.* 1986, Müller *et al.* 1986). Assuming that all XX males are positive for serological H-Y (and there is no exception so far, see Wachtel 1983), presence of the *TDF* region and expression of this antigen would be correlated. From its chromosomal location as well as from its presumed function, the sequence of Page *et al.* (1987) could be part of or identical with the postulated Y-linked gene, exerting a positive control function on serological H-Y antigen (Wolf 1978). This control function could be exerted directly on the structural gene for serological H-Y, or indirectly by including several steps. Another possibility is that the presumed TDF gene and the serological H-Y controlling gene are separate entities located in the same chromosomal region of the Y chromosome.

Corresponding to this Y-linked gene controlling serological H-Y antigen, in my model an X-linked gene is postulated, acting antagonistically, i.e. suppressing serological H-Y activity in a dose-dependent way. This gene has been mapped on human Xp2.23 (Wolf *et al.* 1980). Interestingly, Page *et al.* (1987) identified a DNA sequence on the short arm of the X chromosome which seems to be very similar to the presumptive *TDF* sequence, raising the possibility that this X-linked sequence is part of or identical with the postulated gene exerting a negative control function on the autosomal structural gene for serological H-Y. However, according to regional mapping, this sequence is assigned now to a more proximal position on Xp, thus most probably excluding that possibility (D. Page, this symposium).

My model also includes a gene for a gonad-specific receptor for serological H-Y antigen. It has been shown that serological H-Y is secreted by testicular Sertoli cells and is therefore available as a soluble factor (Müller *et al.* 1978*a*, *b*; Brunner *et al.* 1984). It is able to bind to the somatic cells of the gonads of both sexes, but not to other cells, and this is most probably because of a gonad-specific receptor for serological H-Y (Müller *et al.* 1978*a*, 1979*a*). It can be imagined that mutations of the receptor gene occur, preventing the binding of antigen to the receptor and thus resulting in sex inversion. The X-linked form of XY gonadal dysgenesis in man is a candidate for this condition (Mann *et al.* 1983), and another candidate is the X*Y female wood lemming (Wiberg *et al.* 1982).

I realize that this is a rather straightforward and simplistic model; it does, however, allow for various modifications, and it still meets most of the data obtained in recent years. It may serve the purpose to do experiments aimed at its falsification.

I conclude with some general hypothetical considerations on the sex-determining mechanism in mammals. These considerations are essentially based on discussions with Professor Luis Izquierdo of the University of Chile.

My main premise is that serological H-Y antigen is instrumental for primary testis differentiation.

1. Up to the indifferent stage of the gonadal anlage and before sex-specific morphogenesis takes place, gonadal cells of both sexes differentiate and produce the receptor for serological H-Y antigen which is specific for these cells and not present on any other cells of the embryo. The serological H-Y receptor is the reason for the bipotency of the gonadal anlage.

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2. *TDF* or a closely linked gene, which is present in XY cells only, controls serological H-Y antigen expression in all somatic cells of the male organism. Therefore the male types positive for this antigen in all somatic cells.

3.1. Sertoli-cell differentiation normally depends on TDF. Serological H-Y antigen is present on Sertoli cells as on other somatic cells, but because Sertoli-cell precursors are endowed with the receptor for serological H-Y, the antigen can bind to it and trigger Sertoli cell differentiation. By this process of self-differentiation, the Sertoli cells become able to produce serological H-Y in larger amounts which are then secreted.

3.2. In XX true hermaphrodites, Y-specific DNA has not been detected (Waibel *et al.* 1987; Page *et al.* 1987). Thus TDF is lacking, but nevertheless Sertoli cells are differentiated and testicular tissue is formed in these cases, and they are positive for serological H-Y. Therefore, expression of the autosomal serological H-Y antigen gene may have become independent of TDF, and once serological H-Y is synthesized, Sertoli-cell differentiation and testicular morphogenesis can take place.

4. Serological H-Y antigen, available as a soluble factor, acts via its gonad-specific receptor, inducing Sertoli cells to form seminiferous cords (and possibly also precursors of Leydig cells to become responsive to gonadotropins).

This model could be modified in several ways. One possibility is that the structural gene for serological H-Y is not controlled by TDF, but by another controlling gene located in the neighbourhood of *TDF*. In this case, the serological H-Y system would depend indirectly on TDF.

To my knowledge, the only obstacle for the serological H-Y hypothesis of gonadal differentiation may be the Sxr′ mutation in the mouse which results in XXSxr′ males (McLaren *et al.* 1984) that, in contrast to XXSxr males, are negative for H-Y antigen as defined by cytotoxic T-cells (Gordon *et al.* 1975) and by a transplantation assay (Simpson *et al.* 1986; Wiberg & Lattermann 1987). Definite results with a serological assay have not yet been obtained, but Dr Ellen Goldberg (this symposium) has evidence that non-gonadal cells of XXSxr′ males are negative for this antigen. Interestingly, Ulf Wiberg of our laboratory and Mark Bradley of the University of Otago have purified and characterized a male-specific protein that so far has been shown to be present in the liver of XXSxr and C57BL/6 (B6) male mice, B6, rat and sheep testis, but absent in B6 female and XXSxr′ male liver and ovaries of B6, rat and sheep. Antibodies raised to the purified sex-specific testis protein cross-react with the mouse male-specific liver protein on Western blots. The testis protein was purified on a female anti-male immunoaffinity column. Therefore it is possible that this protein is serological H-Y antigen (U. Wiberg and M. Bradley, personal communication).

These data may be interpreted as falsifying the hypothesis on the role of serological H-Y antigen in gonadal differentiation. However, to me it seems premature to give such an interpretation based on the present results. It cannot be excluded that serological H-Y antigen is present only during a certain time frame, such as the sensitive period of gonadal differentiation; or at the appropriate place, such as the milieu of the testicular Sertoli cells during this sensitive period. In this case, the Sxr′ mutation would affect the spatiotemporal regulation of the serological H-Y gene. Another possibility is that the serological H-Y protein has changed in such a way by this mutation, that it has escaped detection by the methods applied so far.

It must be envisaged that, if serological H-Y antigen is lacking entirely in the XXSxr′ and XSxr′ males, it would be neither necessary nor sufficient for primary male gonadogenesis. In this

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case it may serve the function of an enhancer of growth or differentiation (Heslop *et al.* 1988), or act on later stages of male differentiation as proposed by Burgoyne (1987).

Until the Sxr′ mutation is analysed closer and defined better, I see no reason to abandon the serological H-Y hypothesis of gonadal differentiation which is so strongly supported by various evidence obtained from different cases throughout the vertebrate phylum.

The sex-determining mechanism of placental mammals appears to be adapted to placentation, and it must have evolved from the more primitive mechanism employing sexual steroid hormones as major inducers of differentiation of the heterogametic gonad. Although in many taxonomic groups of non-eutherians serological H-Y is controlled by sexual steroids, in eutherians it may have come under the control of TDF. It can be debated whether, in non-eutherians, TDF has any function at all. However, from lower to higher vertebrates there appears to exist a decreasing responsiveness of indifferent gonadal cells to sexual steroids, whereas sex-determining genes like *TDF* appear to gain more influence. Thus in some fish species, sex can be inverted in either direction by the respective steroid hormone characteristic for the opposite sex. In amphibians, sex inversion can be induced experimentally only in one direction, from the homogametic to the heterogametic sex, but the sex inversion is still complete. In birds, sex inversion is also unidirectional, but transitional and incomplete (ovotestis). Finally, in eutherians sexual steroids remain without effect on primary gonadogenesis. It will be interesting to know if the presumptive *TDF* sequence, which can still be traced in birds (Page *et al.* 1987), is also preserved in lower vertebrates.

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Discussion

M. Adinolfi (*Guy's Hospital, London, U.K.*). I have detected great variations of the levels of serological H-Y antigen in human peripheral white blood cells from male and female subjects. In fact, in agreement with other investigations, I have observed – by using different techniques, including ELISA tests – an overlap of the H-Y values in about 5% of normal males and females. Is this Professor Wolf's experience as well? He seems to suggest that the serological H-Y is either present or absent. I think that small amounts of H-Y molecules can always be detected in normal females.

U. Wolf. We did not test larger samples of normal male and female subjects for inter-individual variation, but in my experience with controls, I have no doubt that such a variation exists. I cannot say, however, if there is an overlap between the ranges of serological H-Y titres of the two sexes, because I did not study this problem systematically. Yamada & Isurugi (1981) found inter-individual variation, but no overlap between sexes.

The question if there is residual serological H-Y activity in normal females, I would answer positively. I have evidence that patients with campomelic dysplasia and XY sex reversal differ from female controls. Although female control tissues usually absorb some anti-H-Y antiserum, tissues derived from these patients practically do not absorb at all. This may be explained by assuming that these patients have a deletion of the H-Y structural gene, whereas in normal females this gene is present.

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U. Wiberg (*Institut für Humangenetik, Freiburg, F.R.G.*). Are there any results from Professor Wolf's laboratory as to the Sxs-status of, especially the testis, Sxr′ mice?

If it should turn out that all Sxr′ mice type Sxs negative, how would Professor Wolf interpret this in light of the hypothesis he presented to us?

U. Wolf. I shall answer these questions together. As I said in my paper, I do not consider the problem of whether or not these mice lack serological H-Y antigen of any kind at all as settled yet. Present evidence indicates that adult animals are negative for H-Y antigen in transplantation and serological tests in non-gonadal tissues. In my view, the following experiments must be performed before a definite answer on the serological H-Y status in this mutant can be given: (1) testing of gonads including embryonic gonads; (2) studies on the expression of the serological H-Y structural gene (which can be done after this gene has been cloned). If the results are negative, the serological H-Y hypothesis of gonadal differentiation must be modified.

Mary F. Lyon, F.R.S. (*M.R.C. Radiobiology Unit, Didcot, U.K.*). In Professor Wolf's studies on inversion experiments and so on, does serological H-Y appear before gonadal differentiation, or after, or can he not tell?

U. Wolf. In our studies on the chicken embryonic gonad, at day 6 of incubation of the eggs the gonads were still at the indifferent stage and they were serological H-Y negative; 12 h later, gonads showed a distinct sex-specific differentiation, and the ovaries were serological H-Y positive whereas the testes remained serological H-Y negative. For technical reasons, we worked with pooled gonads, and because the stages of gonadal development in individual embryos varied to some extent, we chose 12 h intervals for testing.

M. W. J. Ferguson (*Department of Cellular and Structural Biology, University of Manchester, U.K.*). What is the evidence that in vertebrates other than non-eutherian mammals, hormones control the sex differentiation? Are hormone experiments really sex-reversal experiments, i.e. downstream of the primary sex-determining mechanism and not the primary sex-determining mechanism?

Using staged embryos (as opposed to embryonic ages) does the appearance of serological H-Y antigen precede gonadal differentiation? If serological H-Y is a morphogen determining male gonadal organization one must demonstrate that it appears before the organization, if it appears at the same time as the organization cause and effect cannot be properly dissected!

U. Wolf. To answer the first question, I have no evidence that in eutherians primary gonadal differentiation is hormonally controlled. However, there is an interesting paper by Vigier *et al.* (1987) showing that anti-Müllerian hormone can induce a freemartin effect. In non-mammalian vertebrates, sexual steroid hormones may be the physiological inducers of the heterogametic gonad (acting via serological H-Y antigen?), but their role is definitely downstream of the primary sex-determining mechanism.

In reply to the second question, see my answer to Dr Lyon.

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