J. Reprod. Fert. (1984) **70**, 473–479 *Printed in Great Britain*

Origin of anti-Müllerian hormone in bovine freemartin fetuses

B. Vigier, D. Tran, L. Legeai, Jacqueline Bézard* and Nathalie Josso

*Unité de Recherches de Génétique Médicale (INSERM), Hôpital Enfants Malades, 75743 Paris cedex 15, and *Institut National de la Recherche Agronomique, Nouzilly, 37380 Monnaie, France*

Summary. The origin of AMH responsible for Müllerian duct regression in bovine freemartins has been reinvestigated, using a sensitive RIA for this hormone. Between 50 and 80 days, Müllerian duct regression occurs simultaneously in males and freemartins. Both twins exhibited high and positively correlated serum AMH concentrations, whereas gonadal *in-vitro* production of AMH and biological anti-Müllerian activity were detectable at a low level only in 2 out of 13 freemartins. In the gonads of approximately half the freemartins after 80 days, seminiferous tubules differentiated and the gonads produced AMH, but the output was very low compared to that of the male twin. These data suggest that regression of Müllerian duct in freemartins is essentially mediated by AMH produced by the testes of the male twin.

Introduction

Anti-Müllerian hormone (AMH), a glycoprotein dimer (Picard, Tran & Josso, 1978) produced by Sertoli cells (Blanchard & Josso, 1974; Tran & Josso, 1982), is responsible for regression of the Müllerian ducts in male fetuses (Jost, 1947). Müllerian duct regression occurs simultaneously in male and female bovine twins, when these are united by chorionic vascular anastomoses, as is the rule in multiple pregnancies in bovids. The female product of such gestations, the sterile freemartin heifer, has been known to cattle farmers for centuries (Hunter, 1779). According to the hormonal theory favoured by Lillie (1917), a circulating hormone (i.e. AMH), produced by the fetal male and transferred to the female twin via the placenta, is responsible for the inhibition of the development of the Müllerian ducts and for the stunting and secondary masculinization of her gonads. This hypothesis is opposed by the short range of effectiveness of AMH demonstrated by Jost's (1947) classical experiments in the fetal rabbit, and by the inability of the testis of human lateral true hermaphrodites to induce regression of the contralateral Müllerian duct (Van Niekerk, 1976). This contradiction can be resolved by assuming that extensive dissemination of AMH is a peculiarity of cattle (Ohno, 1979).

As an alternative to the hormonal theory outlined above, the possibility that the freemartin's own gonads participate in the regression of her own Müllerian ducts has been considered (Lillie, 1917; Witschi, 1939; Jost, Vigier & Prépin, 1972; Vigier, Picard, Bézard & Josso, 1981). Morphological masculinization of freemartin gonads, indicated by the appearance of seminiferous tubules, is a relatively late event (Jost, Vigier, Prépin & Perchellet, 1973) but may be preceded by signs of functional virilization, such as testosterone production (Shore & Shemesh, 1981) or anti-Müllerian activity (Vigier *et al.*, 1981). The application of monoclonal antibody-derived technology (Vigier, Legeai, Picard & Josso, 1982; Tran & Josso, 1982) to the study of AMH has led us to reinvestigate the cause of Müllerian regression in the freemartin fetus.

0022-4251/84/020473-08$02·00/0

Materials and Methods

Bovine material

Nine heterosexual litters <80 days of gestation were obtained by superovulation (Mauléon. Mariana, Benoit, Solari & Chupin, 1970) or blastocyst transfer (Renard, Ozil & Heyman, 1981) from the Institut National de la Recherche Agronomique at Nouzilly and Jouy en Josas; the gestational age was known exactly. Five litters were composed of 1 male and 1 female, two of 1 male and 2 females, and two of 3 males and 2 females. The presence of vascular anastomoses between the fetuses was checked routinely. Freemartin effects upon ovaries and Müllerian ducts were detectable in fetuses over 51 days. Fifteen heterosexual pairs of twins between 80 and 166 days were obtained from the Rouen slaughterhouse; their gestational age was estimated from their crown–rump length (Maneely, 1952). Inhibition of ovarian and Müllerian growth was evident in all females; in addition, 4 showed slight virilization of the external genitalia. Male and female singletons were obtained from the INRA or the Rouen slaughterhouse.

AMH radioimmunoassay

AMH was measured by RIA as previously described (Vigier *et al.*, 1982) in fetal serum and in incubation media in which fragments of gonadal tissue has been maintained for 24 h (Vigier, Tran, du Mesnil du Buisson, Heyman & Josso, 1983). Culture medium consisted of Eagle's MEM, with 10% bovine female fetal serum added. Results are expressed as mU AMH per ml serum or released per mg gonadal tissue. One unit of AMH is the amount released by 1 g of fetal testicular tissue incubated *in vitro* over 4 h. The RIA measures AMH concentrations as low as 0·1–0·2 mU/ml, with a between-assay variability under 5%, validated for values between 2 and 60 mU/ml.

AMH immunocytochemistry

Freemartin gonads were treated immunocytochemically for AMH by the method previously described for bovine testicular tissue (Tran & Josso, 1982), except that the fixative described by McLean & Nakane (1974) was employed and an avidin–biotin method (Hsu, Raine & Fanger, 1981) was substituted for the immunoperoxidase technique.

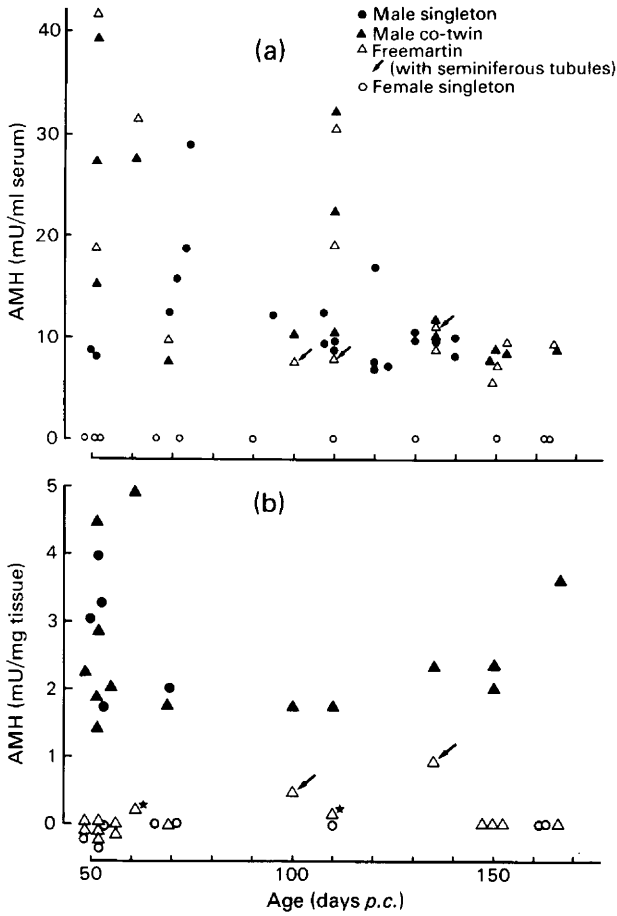
Bioassay for AMH and histology in freemartin gonads

Bioassay for AMH was performed according to Picon (1969) using Müllerian ducts from 14·5-day-old fetal rats as the target organ. Assessment of the degree of regression of Müllerian ducts exposed for 3 days in organ culture to gonadal tissue was performed as previously described (Josso, Forest & Picard, 1975). Because of the limited amount of tissue available, gonadal fragments were first maintained for 24 h in culture medium, to allow assay of the AMH released, and then associated with fetal rat reproductive tracts. At the end of the culture period, the explants were serially sectioned and gonadal tissue from freemartins was carefully examined for the presence of seminiferous tubules.

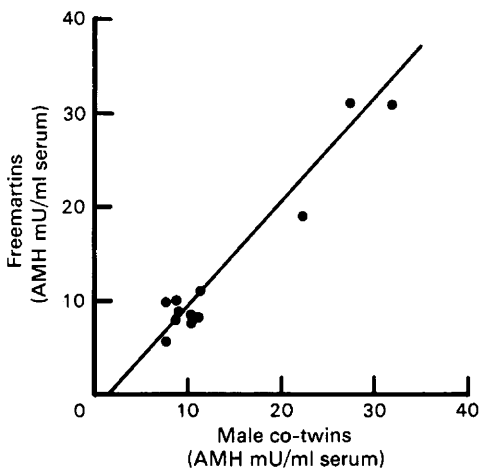
Results

Serum AMH concentrations

Serum AMH was measured by RIA in freemartins and their male twins, and also in male and female singletons. Individual results are shown in Text-fig. 1(a) and Table 1, and mean values according to age, sex and pregnancy status in Table 2. From 50 to 110 days, male and freemartin fetuses exhibited high serum AMH concentrations. Individual variations were wide, but, within



Text-fig. 1. Concentrations of AMH in the serum of bovine fetuses (a), and the production of AMH by gonadal fragments incubated *in vitro* over 24 h (b). *Value calculated using the calibration curve beyond the limit of its published validation.



Text-fig. 2. Relationship between serum AMH concentration in freemartins and their male twins: $y = 1{\cdot}045\ x - 1{\cdot}3$ ($r = 0{\cdot}969$).

Table 1. Serum and gonadal AMH in freemartin fetuses

Freemartins				Gonad					
				Histology			Anti-Müllerian activity (no. of rat reproductive tracts with Müllerian regression)		
Litter	Age (days *p.c.*)	Fetus	Serum AMH conc. (mU/ml)	Seminiferous tubules	Immuno-reaction for AMH	In-vitro production of AMH (mU/mg tissue)	Complete	Incomplete	None
N12†	49	1		–		ND	0	0	4
		5		–		ND	0	0	2
N11	49	2		–			0	0	3
N15	51	1		–	–	ND	0	0	4
N23†	51	4	42·7		–	ND			
		5	18·7		–	ND			
N14‡	55	1		–		ND	0	0	4
		2		–		ND	0	0	5
N08‡	61	2		–			0	2	0
		3		–			0	0	2
N24	61	2	31·7	–	–	0·2*	0	1	1
N22	69	2	9·7	–	–	ND	0	0	4
R21	80	2		–			0	0	2
R04	95	2		–			0	0	4
R37	100	2	7·5	+		0·5	0	1	4
R17	110	2	7·8	+ +			0	4	2
R40	110	2	19·1	–	–		0	0	5
R42	110	2	30·7	–	–	0·1*	0	0	4
R06	130	2		+ +			1	6	1
R12	130	2		–			0	0	4
R43	135	2	11·2	+ +	+	0·9	0	2	4
R45	135	2	8·9						
R32	150	2		+ +			0	6	0
R41	150	2	7·7	–	–	ND	0	0	5
R44	150	2	5·6	–	–	ND	0	0	5
R47	150	2	9·9	–	–	ND	0	0	5
R23	160	2		+			0	0	5
R36	165	2	8·8	–		ND	0	0	6

N, obtained from Nouzilly (INRA), age known exactly; R, obtained from Rouen slaughterhouse, age deduced from crown–rump length; ND, not detectable.

* Value calculated using the calibration curve beyond the limits of its published validation.

† Quintuplets (3 males).

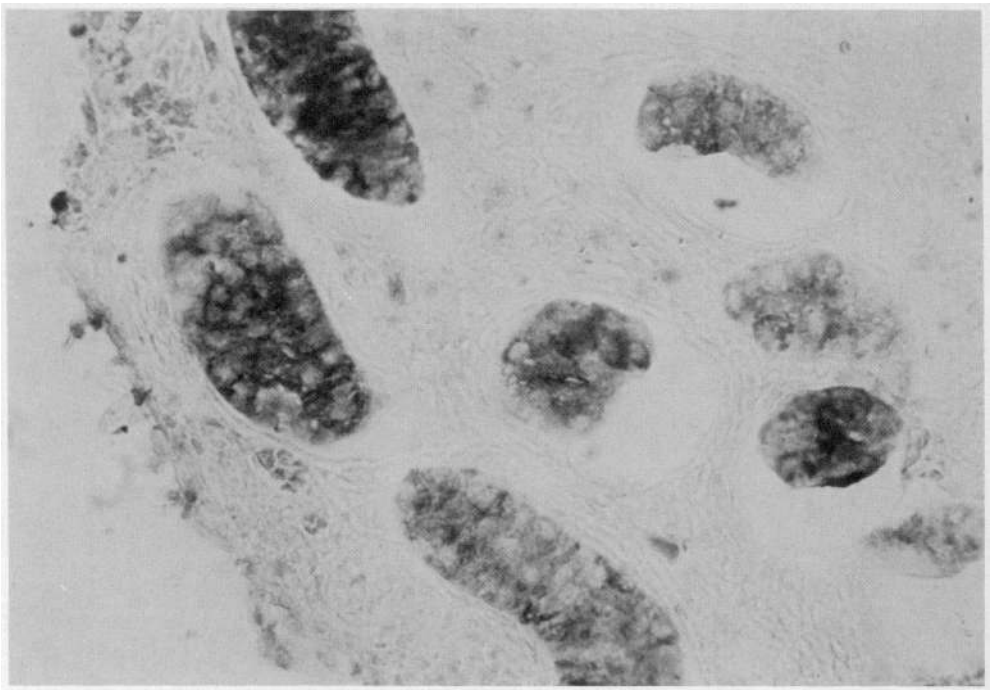
‡ Triplets (1 male).

each litter, a positive correlation ($r = 0{\cdot}97$) was demonstrated between values in twins of either sex (Text-fig. 2). However, the presence of seminiferous tubules within the gonads of a freemartin had no apparent relationship to the concentration of AMH in its serum (Text-fig. 1a).

In-vitro production of AMH by gonadal tissue

The production of AMH by fragments of testicular and ovarian tissue cultured for 24 h was measured by RIA. Individual results are shown in Text-fig. 1(b) and Table 1; mean results according to age, sex and pregnancy status are presented in Table 2. Fetal testes of singletons and twins to freemartins produced large amounts of AMH between 50 and 65 days, the amount of AMH produced per unit weight decreasing slightly in the later stages of pregnancy. However, AMH production by freemartin gonads could be detected only in 1 culture out of 9 before 80 days, and in 3 out of 7 cultures after 80 days. In 2 of the latter, AMH production was associated with the presence

PLATE 1



Immunocytochemical localization of AMH in the gonad of a 135-day-old freemartin (R43 F2). Immunoreactive AMH is located within the few seminiferous cords present in the gonad. Cryostat sections (10 μm), avidin–biotin peroxidase technique; × 500.

(*Facing p.* 476)

Table 2. Serum and testicular AMH in bovine fetuses according to their age, sex and gestational status

Fetuses			Serum AMH conc. (mU/ml)	Gonad			
				In-vitro production of AMH (mU/mg tissue)	Anti-Müllerian activity (no. of rat reproductive tracts with Müllerian regression)		
Age (days *p.c.*)	Gestational status	No. studied			Complete	Incomplete	None
49–80*	Male singletons	11	14·68 ± 8·54	2·80 ± 0·90	—	—	—
	Male twins	13	23·14 ± 12·16	2·78 ± 1·18	43	4	0
	Freemartins	13	25·70 ± 14·50	0–0·2	0	3	31
	Females	6	0	0	—	—	—
81–166†	Male singletons	14	11·50 ± 5·14	—	—	—	—
	Male twins	15	12·30 ± 8·72	2·06 ± 0·88	12	36	2
	Freemartins	15	11·66 ± 7·64	0–0·9	1	19	54
	Females	6	0	0	—	—	—

Values are mean ± s.d.
* Fetuses obtained from INRA; age known exactly.
† Fetuses obtained from Rouen slaughterhouse; age calculated.

of seminiferous tubules within the gonad. The amount of AMH produced by gonadal tissue of freemartins, irrespective of their age, was always low, in 2 cases barely detectable, and could never be compared with the level of AMH production by testicular tissue. No AMH production by ovarian tissue of normal females could be demonstrated at any age.

AMH immunocytochemistry

Only the results obtained for freemartins are reported (Table 1) since those pertaining to normal bovine fetuses of both sexes have been published previously (Tran & Josso, 1982; Vigier *et al.*, 1983). Gonads from 11 freemartins were studied by this technique, but only one contained seminiferous tubules which stained positively for AMH (Plate 1).

Anti-Müllerian activity

Bioassay for anti-Müllerian activity was carried out with gonadal tissue from males and freemartins from 22 heterosexual litters, the data for 6 of which have been previously published (Vigier *et al.*, 1981). The results are shown in Table 1 for freemartins and their male twins in Table 2. The level of biological anti-Müllerian activity was highest in males under 80 days, and slightly lower in older ones. Slight anti-Müllerian activity was exhibited by the gonads of 2 out of 11 freemartins between 49 and 80 days, and by those of 5 out of 14 older ones, all of which contained seminiferous tubules.

Discussion

Our results indicate that serum AMH in freemartins is constantly elevated and closely correlated with the level observed in the male twin, the acme coinciding with the period of physiological Müllerian regression in the normal male fetus and in freemartins (Jost *et al.*, 1972), i.e. 50 to 80 days

of gestation. During that period, testes actively produce AMH, but freemartin gonads only rarely and in limited amounts. At first glance, production of AMH by young freemartin gonads in which seminiferous tubules have not yet appeared may seem surprising, since in the normal testis Sertoli cells begin to produce AMH at the time they organize into seminiferous tubules (Tran, Meusy-Dessolle & Josso, 1977). However, experimental dissociation between the 2 maturational events has been demonstrated (Magre, Jost & Valentino, 1982), and could also explain the exceptional cases in which AMH production by the freemartin gonad precedes the appearance of organized seminiferous tubules.

After 80 days, evidence for AMH production by the freemartin gonad was obtained in approximately half the cases studied, and was usually associated with the presence of seminiferous tubules. Differentiation of seminiferous tubules within the freemartin gonad has been successively attributed to the presence of XY cells in the gonadal primordium (Ohno, Christian, Wachtel & Koo, 1976) or to passage of H-Y antigen from the male to the female twin (Wachtel, Hall, Müller & Chaganti, 1980). The amount of AMH produced by the seminiferous tubules scattered within the freemartin gonad is negligible compared to the amount released by the testis of the male twin, and does not significantly increase serum AMH in these animals.

In a previous study (Vigier *et al.*, 1981), we had questioned the testicular origin of the AMH responsible for Müllerian regression in the freemartin because of our failure, in most cases, to extract anti-Müllerian activity from the serum of freemartins or their male twins. Using a more reliable and sensitive method, we have now demonstrated the presence of circulating AMH at similar concentrations in the serum of twins of both sexes, and have shown that at the time of physiological regression of Müllerian ducts, the freemartin gonad produces AMH only rarely and in minute amounts.

These results may therefore be taken as confirmation of the classical 'hormonal theory', regarding the regression of Müllerian derivatives in freemartin fetuses. Whether the same product is also responsible for ovarian stunting, an event chronologically and quantitatively correlated with Müllerian regression (Jost *et al.*, 1972), remains to be determined. Interruption of placental anastomoses between the male and the female twin before 45 days of gestation prevents Müllerian regression and ovarian stunting in the female (Vigier, Prépin & Jost, 1976), but this does not prove that the same substance is involved in both effects. Wachtel *et al.* (1980) have presented evidence for circulating H-Y antigen in the freemartin and have postulated that H-Y antigen of testicular origin is responsible for the masculinization of its ovary. However, Wachtel *et al.* (1980) did not state whether this explanation applies only to the active phase of masculinization, i.e. the morphological and functional differentiation of Sertoli cells which occurs in approximately half the freemartins over 80 days (Jost *et al.*, 1973), or whether H-Y antigen also bears responsibility for the initial stunting and germ cell death which always affect the ovary of the female twin. Further work is also needed to understand why the serum AMH concentration is similar in male singletons and in male twins to freemartins. Obviously, the volume of distribution for the AMH produced by the testes of male twins is much larger, while testicular weight (Jost *et al.*, 1972) and testicular AMH production do not appear to vary according to pregnancy status. Study of the regulation of AMH secretion by fetal Sertoli cells may resolve this apparent contradiction.

We thank D. Chupin, Y. Heyman, P. Chesné, V. Garnier of the Institut National de la Recherche Agronomique at Nouzilly and Jouy en Josas, and M. Lahure of the slaughterhouse at Rouen for the calf fetuses; A. Simon and V. Ravet for histological sections; and A. Ardouin-Dumazet for typing the manuscript.

References

Blanchard, M.G. & Josso, N. (1974) Source of the anti-Müllerian hormone synthesized by the fetal testis: Müllerian-inhibiting activity of fetal bovine Sertoli cells in tissue culture. *Pediatr. Res.* **8**, 968–971.

Hsu, S.M., Raine, L. & Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. *J. Histochem. Cytochem.* **29**, 577–580.

Hunter, J. (1779) Account of the freemartin. *Phil. Trans. R. Soc.* **69**, 279–293. (Cited by Wachtel *et al.*, 1980.)

Josso, N., Forest, M.G. & Picard, J.Y. (1975) Müllerian-inhibiting activity of calf fetal testis: relationship to testosterone and protein synthesis. *Biol. Reprod.* **13**, 163–167.

Jost, A. (1947) Recherches sur la différenciation sexuelle de l'embryon de lapin. III. Rôle des gonads foetales dans la différenciation sexuelle somatique. *Archs Anat. microsc. Morph. exp.* **36**, 271–315.

Jost, A., Vigier, B. & Prépin, J. (1972) Freemartins in cattle: the first steps of sexual organogenesis. *J. Reprod. Fert.* **29**, 349–379.

Jost, A., Vigier, B., Prépin, J. & Perchellet, J.P. (1973) Studies on sex differentiation in mammals. *Recent Prog. Horm. Res.* **29**, 1–41.

Lillie, F.R. (1917) The freemartin, a study of the action of sex hormones in foetal life of cattle. *J. exp. Zool.* **23**, 371–452.

Magre, S., Jost, A. & Valentino, O. (1982) Morphogénèse testiculaire *in vitro* et différenciation fonctionnelle des cellules de Sertoli. Influence du sérum de foetus de veau. In *Ontogénèse du Système Endocrinien* p. 556, abstr. Eds J. M. Saez, J. Bertrand, J. R. Ducharme & R. Collu. INSERM, Paris.

Maneely, R.B. (1952) Note on the ageing of bovine embryos. *Vet. Rec.* **35**, 509–511.

Mauléon, P., Mariana, J.C., Benoit, M., Solari, A. & Chupin, D. (1970) Influence de différentes doses de PMSG et HCG injectés en phase folliculaire du cycle oestrien, sur le nombre et le rendement d'ovulations de vaches de race Française Frisonne pie noire. *Annls Biol. anim. Biochim. Biophys.* **10**, 31–46.

McLean, I.W. & Nakane, P.K. (1974) Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22**, 1077–1083.

Ohno, S. (1979) *Major Sex-Determining Genes*. Springer-Verlag, Heidelberg.

Ohno, S., Christian, L.C., Wachtel, S.S. & Koo, G.C. (1976) Hormone-like role of H-Y antigen in bovine freemartin gonad. *Nature, Lond.* **261**, 597–599.

Picard, J.Y., Tran, D. & Josso, N. (1978) Biosynthesis of labelled anti-Müllerian hormone by fetal testes: evidence for the glycoprotein nature of the hormone and for its disulfide-bonded structure. *Molec. cell. Endocr.* **12**, 17–30.

Picon, R. (1969) Action du testicule foetal sur le développement *in vitro* des canaux de Müller chez le rat. *Archs Anat. microsc. Morph. exp.* **58**, 1–19.

Renard, J.P., Ozil, J.P. & Heyman, Y. (1981) Cervical transfer of deep frozen cattle embryos. *Theriogenology* **15**, 311–320.

Shore, L. & Shemesh, M. (1981) Altered steroidogenesis by the fetal bovine freemartin ovary. *J. Reprod. Fert.* **63**, 309–314.

Tran, D. & Josso, N. (1982) Localization of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody. *Endocrinology* **111**, 1562–1567.

Tran, D., Meusy-Dessolle, N. & Josso, N. (1977) Anti-Müllerian hormone is a functional marker of foetal Sertoli cells. *Nature, Lond.* **269**, 411–412.

Van Niekerk, W.A. (1976) True hermaphroditism. Analytic review with a report of 3 new cases. *Am. J. Obstet. Gynec.* **126**, 890–907.

Vigier, B., Prépin, J. & Jost, A. (1976) Chronologie du développement de l'appareil génital du foetus de veau. *Archs Anat. microsc. Morph. exp.* **65**, 77–102.

Vigier, B., Picard, J.Y., Bézard, J. & Josso, N. (1981) Anti-Müllerian hormone: a local or long distance morphogenetic factor? *Hum. Genet.* **58**, 85–90.

Vigier, B., Legeai, L., Picard, J.Y. & Josso, N. (1982) A sensitive radioimmunoassay for bovine anti-Müllerian hormone, allowing its detection in male and freemartin fetal serum. *Endocrinology* **111**, 1409–1411.

Vigier, B., Tran, D., du Mesnil du Buisson, F., Heyman, Y. & Josso, N. (1983) Use of monoclonal antibody techniques to study the ontogeny of bovine anti-Müllerian hormone. *J. Reprod. Fert.* **69**, 207–214.

Wachtel, S.S., Hall, J.L., Müller, U. & Chaganti, R.S.K. (1980) Serum-born H-Y antigen in the fetal bovine freemartin. *Cell* **21**, 917–926.

Witschi, E. (1939) Modification of the development of sex in lower vertebrates and in mammals. In *Sex and Internal Secretions*, 2nd edn, pp. 145–226. Eds E. Allen, C. Danforth & E. A. Doisy. Williams and Wilkins, Baltimore.

Received 1 July 1983