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Original Article

Expression of sex-specific molecular markers in clones of bipartite allophenic nemertines produced by somatic embryogenesis from *Lineus sanguineus* male/female chimera fragments

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ABSTRACT SDS-PAGE electrophoresis showed major sex-specific proteins in sexually maturing and mature *Lineus sanguineus*. These "egg-specific" (145, 78 and 40 kDa) and "sperm-specific" (55, 52 and 28 kDa) proteins are useful for studying sex differentiation in bilaterally allophenic worms produced by asexual reproduction of bipartite male/female chimeric worms. This study was carried out on 2 symmetrical clones of bilaterally allophenic worms, derived by somatic embryogenesis from fragments transected from chimeras obtained by exchange-grafting lateral body halves of male and female specimens, and from their asexually-derived progeny. The electrophoretic patterns of proteins extracted from sexually immature, maturing and mature allophenic animals from the 5th to the 19th year of cloning, showed the presence of all female-specific markers and the absence of male-specific markers. There was also complete biochemical feminization of the male halves. The synthesis of the only egg-specific molecules in initially male lateral body halves means that the long-term cloning results in the total repression of genes encoding sperm-specific proteins, since genetically male determinant-bearing cells can randomly re-express the testis characteristic as fertile but rudimentary male gonads.

KEY WORDS: *sex-specific markers, sex differentiation, chimera cloning, allophenic nemertines, somatic embryogenesis*

Introduction

The difficulties that usually prevent successful construction of animals by piecing together body parts from several organisms can be overcome by using nemertines of the genus *Lineus*. Such chimeras are useful for studying various aspects of adult metazoan development, such as regeneration, somatic embryogenesis from fragments and differentiation of sex characters. They are produced by grafting together male and female components of *L. sanguineus* worms. Their ability for asexual reproduction allowed the production of clones of bilaterally allophenic animals from bipartite male/female chimeras (Sivaradjam and Bierne, 1980). The first bipartite chimeras were constructed in 1975 by grafting the lateral halves from a phenotypically dark-brown male onto the anatomically complementary halves of a phenotypically light-brown female (Fig. 1). Since this year, 2 stable laboratory strains of bilaterally allophenic nemertines have been continued by somatic embryogenesis from these 2 bipartite chimeras constructed with symmetrical worm halves of opposite sexes (Sivaradjam and Bierne, 1980, 1981). The bipartite cutaneous phenotypes have been transmitted and expressed during the asexual reproduction of each clone (Fig. 2A).

All Lineus are strictly gonochoric animals. At the onset of each period of sexual maturation (annually in nemertines), short ducts grow out from gonads to open at the body surface. In *L. sanguineus* the oviducts of females occupy the same laterodorsal position as do the vasa deferentia from the male testes. At maturity there are no external differences in the location of the gonadopores of the 2 sexes: the genital pores form a dorsolateral row down each side of the body, so that males and females are indistinguishable without gonad examination. Figure 2B shows a mature allophenic worm with a dorsolateral row of genital pores.

For short-term cloning of bipartite heterosexual chimeras, sexual differentiation starts in allophenic worms with a transient stage of gonad developmental autonomy (primary gynandromorphous effect) and ends by complete feminization at sexual maturity. This sex reversal (feminization of the male component) is always secondary during the first 3 cloning years, but became primary from the 5th cloning year (Sivaradjam and Bierne, 1989). These results

Abbreviations used in this paper: Chaps, cholamidoprolyldmethylhydroxypropane sulfonate; DTT, dithiotreitol; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS.

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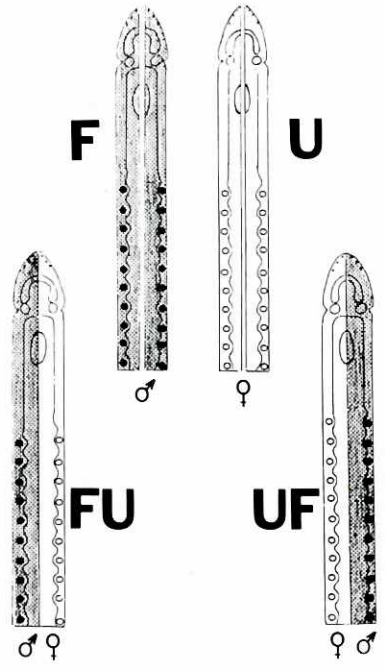

Fig. 1. *Lineus sanguineus* allogenic chimeras FU and UF. *Two symmetric allogencic chimeras, FU and UF, were constructed by grafting the lateral halves from a phenotypically dark-brown male (F, France) onto the anatomically complementary halves from a phenotypically light-brown female (U, Uruguay). Thus, the 2 halves of each heterosexual chimera formed by grafting the right half of one worm onto the left half of a worm of the opposite sex had different colors. The FU chimera is mirror image of the UF chimera. Dark circles represent testis, and light circles ovaries. (Modified from Sivaradjam and Bierne, 1989).*

are based on histological studies for 15 years (Sivaradjam and Bierne, 1981, 1985, 1989). Several proteins have recently been found in sexually mature *Lineus* (Bierne *et al.*, 1993). SDS-PAGE studies of total protein extracts from *L. sanguineus* worms at various stages of sexual maturation revealed several major sex-specific proteins. These molecular sex markers have been used to investigate the biochemical sex phenotype in long-term clones of allophenic *Lineus* and so complement the histological investigations.

Results

Biochemical sex dimorphism

The protein pattern changed considerably during *L. sanguineus* sexual maturation (Fig. 3). SDS-polacrylamide gel electrophoresis of worm extracts of the 2 clones, U1 (females) and C_4 (males), showed the presence of several sex-specific proteins in both maturing and mature females, and in maturing and mature males. A major protein (145 kDa) and 2 minor proteins (78 and 40 kDa) were present only in females since these markers were absent from patterns from males at all stages of sexual maturation. Three male-specific bands (55, 52 and 28 kDa) were identified only in maturing and mature male electrophoretic patterns. Three low molecular weight non-sex-specific proteins were also more abundant in mature than in immature males, suggesting an increasing synthesis of these proteins during spermatogenesis.

The organismal locations of these sex-specific proteins were determined by comparing the proteins of gonad contents emitted by mature worms with the proteins of immature, mature or post-mature (after partial or complete emission of eggs or sperm) animals (Figs. 3 and 4). The female sex-specific molecules were found in the eggs (Figs. 3 and 4A) and the male sex-specific molecules were found in the sperm (Figs. 3 and 4B).

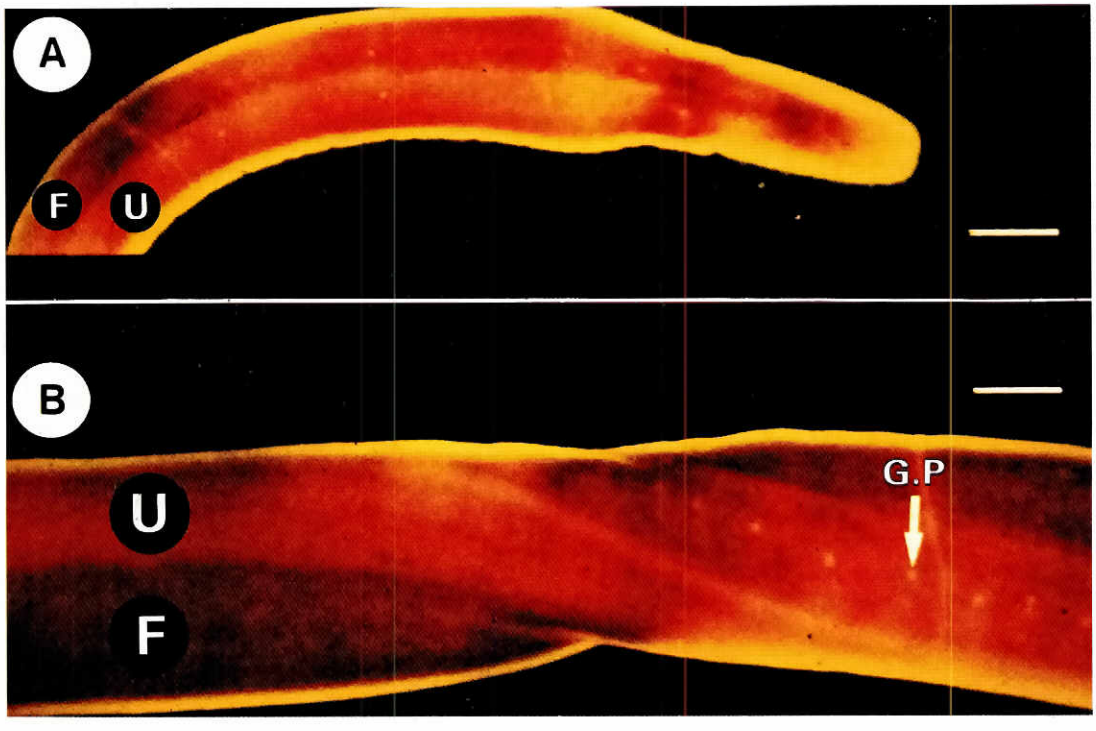

Fig. 2. An allophenic worm photographed to show the persistence of the difference in pigmentation on the 2 sides of the body 19 years after cloning. (A) *Dorsal view of the allophenic nemertine from the FU clone showing the transmission and expression of the bipartite cutaneous phenotype for long-term asexual reproduction. Bar, 500 µm.* **(B)** Ventral *and dorsal view of a sexually mature allophenic worm, where genital pores (g.p) (arrow) form a dorsolateral row. Bar, 250 µm.*

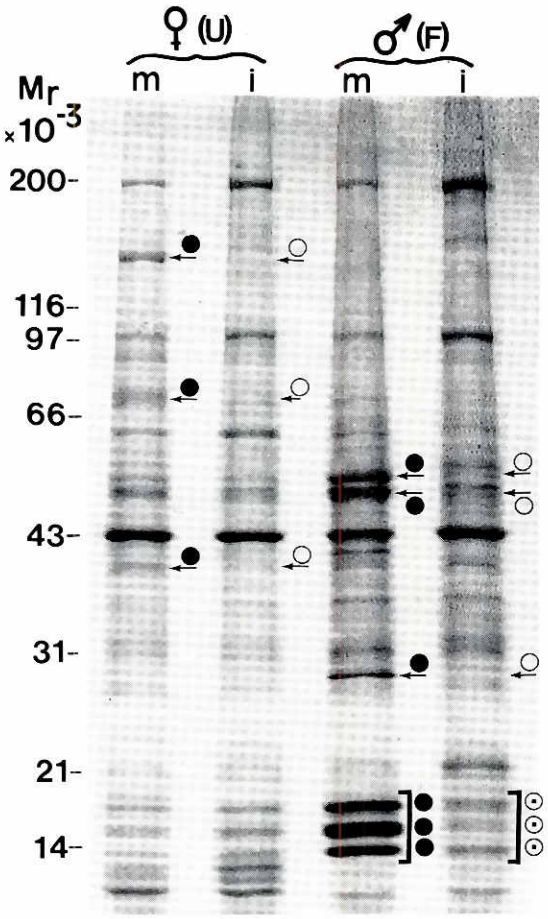

Fig. 3. Electrophoretic pattern of proteins extracted from immature (i) and mature (m) *Lineus sanguineus* females (U) and males (F). *Proteins (40 µg per lane) were separated by SDS-PAGE and stained with Coomassie blue. The samples in each lane are identified on the figure. The positions of sex-specific proteins are marked with arrows. Black symbols indicate protein present, white symbols show absence of protein and pointed white symbols show small amounts of protein. Left lane, position of molecular weight markers.*

These proteins are present only in sexually maturing and mature worms, and absent from immature or complete post-mature animals. They form the base of the biochemical sexual dimorphism. These sex-specific "yolk proteins" (145, 78 and 40 kDa) or "sperm-specific proteins" (55, 52 and 28 kDa), can be used in monitoring cell lineages and in the investigation of cell interactions, especially in the sexual differentiation occurring in clones from asexual multiplication of heterosexual nemertine chimeras.

Feminization of allophenic nemertines

The 'ovary' characteristic alone is expressed on the 2 sides of the allophenic worm body of long-term organism clones (from 5th year to date, i.e. 19 years) (Sivaradjam and Bierne, 1981, 1985, 1989). The histological feminization was confirmed by the protein profile. Electrophoretic analysis of immature and mature heterosexual cloned FU (Fig. 5A) as well as UF (Fig. 5B) worms showed the presence of all female sex-specific proteins in the 2 halves (no sperm-specific protein was identified) and the total feminization of the initial male halves (left in FU worms and right in UF worms). The electrophoretic patterns from the isolated right and left halves were the same as the pattern from the whole worm. Oocytes that developed in 'male' halves were not distinguishable from oocytes that differentiated in female halves. The perfect symmetry of the protein constituents of the FU and UF allophenic worms is shown in Fig. 6. The present electrophoretic study of egg proteins showed the biochemical feminization of male halves. By contrast, the male pigmentation of "F" halves of allophenic worms contrasted with the female pigmentation of the "U" halves.

From the 5th year of cloning up to date, the differentiation of the single 'ovary' characteristic was the usual type of allophenic worm gonadogenesis. However, histological examination of some worms from a sub-clone FU called FUt showed that the right halves (female for FU) contained oocytes whereas the left halves (male for FU) were sterile, except in a few intestinal diverticles where very young testes were found (Fig. 7). FUt worms had a singular pattern of proteins, the male halves were immature (the early development and small number of these testes prevented detection "sperm-

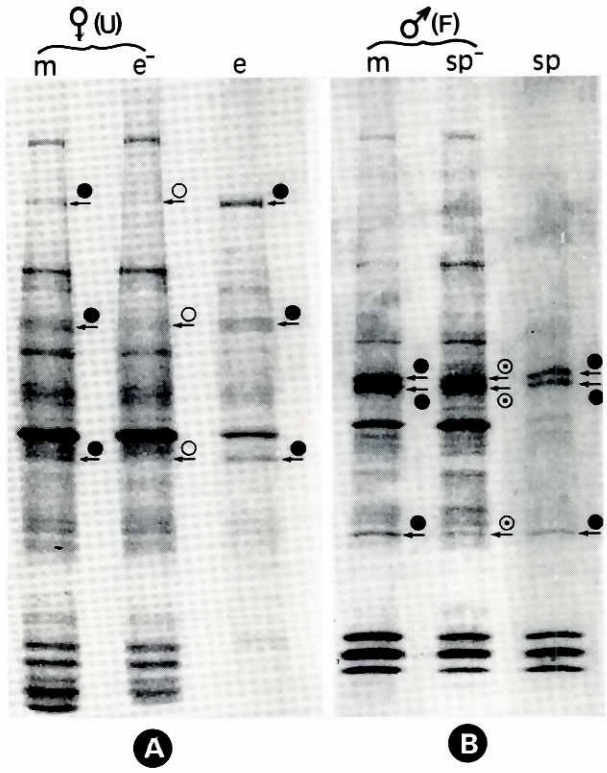

Fig. 4. Electrophoretic pattern of proteins from a female (U) and a male (F) *Lineus sanguineus*. *Proteins (40 µg per lane) were separated by SDS-PAGE and stained with Coomassie blue. The samples in each lane are identified on the figure.* **(A)** *Mature female (m), mature female in which eggs were totally extracted (e-), and extracted eggs (e).* **(B)** *Mature male (m), mature male in which sperm was partially extracted (sp-), and extracted sperm (sp). The positions of sex-specific proteins are marked with arrows. Black symbols indicate protein present, white symbols show absence of protein and pointed white symbols show small amounts of protein.*

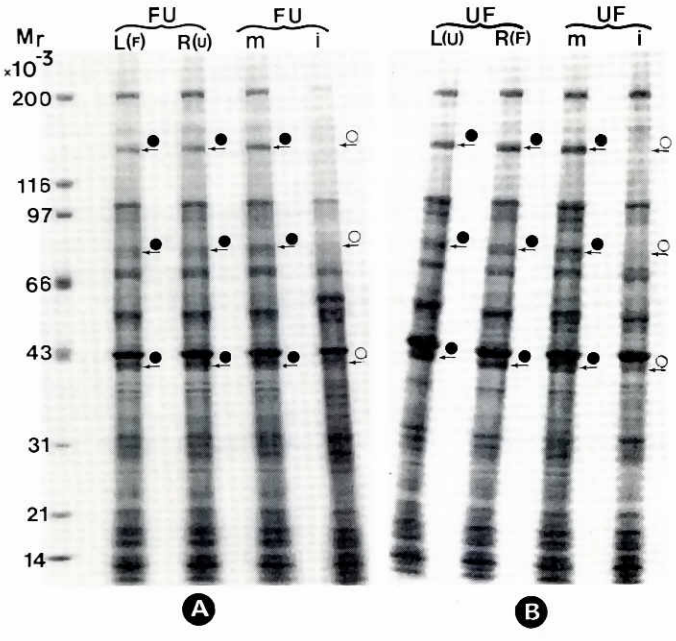

Fig. 5. Electrophoregrams of proteins extracted from a bipartite allophenic *Lineus sanguineus* in the 19th year of cloning. *Proteins (40 µg per lane) were separated by SDS-PAGE and stained with Coomassie blue.* **(A)** *FU clone specimens: patterns from the left half, initially male (L_F), and the right half, initially female (R_U), of a mature worm are compared with patterns from the whole mature (m) and the whole immature (i) worms.* **(B)** *UF clone specimens: patterns from the left half, initially female (L_U), and the right half, initially male (R_F), of a mature worm are compared with patterns from whole mature (m) and whole immature (i) worms. The samples in each lane are identified on the figure. The positions of sex-specific proteins are marked with arrows. Black symbols indicate protein present, white symbols show absence of protein. Left lane: position of molecular weight markers. At sexual maturity, initially male halves of both clones had only female sex-specific proteins. No sperm-specific proteins were detected.*

specific proteins" in electrophoregrams), whereas female sex-specific proteins were abundant in the female halves. This confirmed the cytological dissymmetry. Furthermore, a few allophenic worms could randomly re-express the 'testis' characteristic as a rudimentary male gonad for the 19th year of asexual multiplication.

Discussion

The differentiation and development of sexual characters has been the subject of experimental research on nemertines for some time (Bierne, 1970, 1990; Rué and Bierne, 1978, 1982, 1988; Sivaradjam and Bierne, 1981, 1989). The identification of sex-specific proteins in several *Lineus* species (Bierne *et al.*, 1993) led to biochemical studies of the sexual differentiation of these gonochoristic marine worms which have special biological features (regeneration and reconstruction by grafting) for the study of sex and reproduction. The use of chimeras has become a promising technique in developmental biology (Le Douarin and McLaren, 1984).

Two clones of allophenic, heterosexual worms were produced by asexual reproduction of 2 *L. sanguineus* bipartite male/female chimeras, i.e. by somatic embryogenesis from a large number of pieces transected from 2 symmetric bilaterally allogencic FU and UF reconstructed worms and their asexually-derived progeny. This clonal reproduction of chimeric *L. sanguineus* allowed us to study the transmission of 2 characters, sex and pigmentation. The bipartite cutaneous pigmentation of chimeras was transmitted to, and expressed in, all worms (bipartite allophenic *Lineus*). But the worms became completely feminized from the 5th year of cloning (Sivaradjam and Bierne, 1981, 1989).

SDS-polyacrylamide gel electrophoresis was used to identify several major sex-specific proteins, from the eggs or sperm of maturing *L. sanguineus* worms. These abundant proteins form the basis for biochemical sex dimorphism. Electrophoretic investigations reveal the presence of female-(egg-) and the absence of male-(sperm-)-specific proteins in mature FU and UF allophenic worms. Feminization is biochemically total from the 5th year of cloning. The biochemical differentiation occurring in the 2 clones is the same. The eventual expression of only egg-specific molecules means that the long-term cloning resulted in either the elimination or complete repression of genes encoding sperm-specific proteins.

The discreet reminiscence of the male phenotype observed in the FUt sub-clone shows that the testis determinant had not been eliminated. The clear difference between the F_t and U_t halves indicates a bilaterally chimeric state of sex characteristics. The present cytological and biochemical data support our hypothesis (Sivaradjam and Bierne, 1987) that heterosexual allophenic worms produced by somatic embryogenesis retain genetically male and female cells, but that interactions between cells of 2 genetic sexes eventually result in complete repression of the testis determinant, and inhibition of sperm-specific gene expression. So phenotypically female FU and UF clones are probably genetically chimeric, i.e. genotypically male and female.

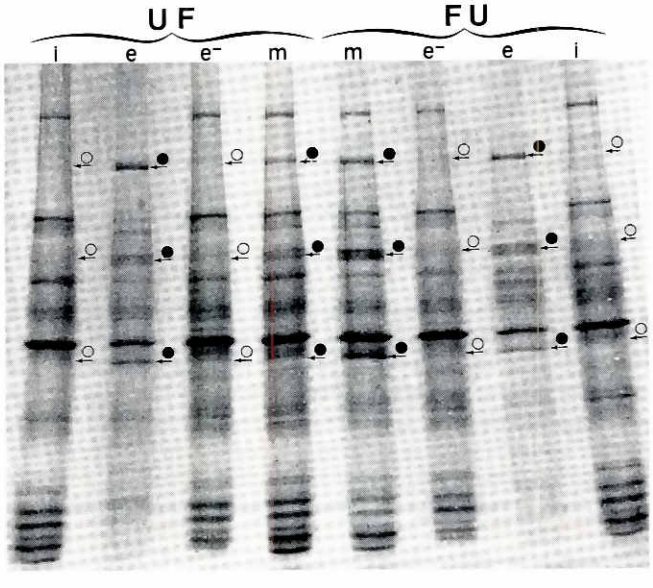

Fig. 6. Electrophoretic pattern of proteins extracted from bipartite allophenic *Lineus sanguineus* FU and UF worms in the 19th year of cloning. *Proteins (40 µg per lane) were separated by SDS-PAGE and stained with Coomassie blue. The samples in each lane are identified on the figure. Immature worm (i), mature worm (m), mature worm from which eggs were extracted (e-), and extracted eggs (e). Note the complete biochemical feminization of allophenic worms. The positions of sex-specific proteins are arrowed. Black symbols indicate protein presence, and white symbols show absence of protein.*

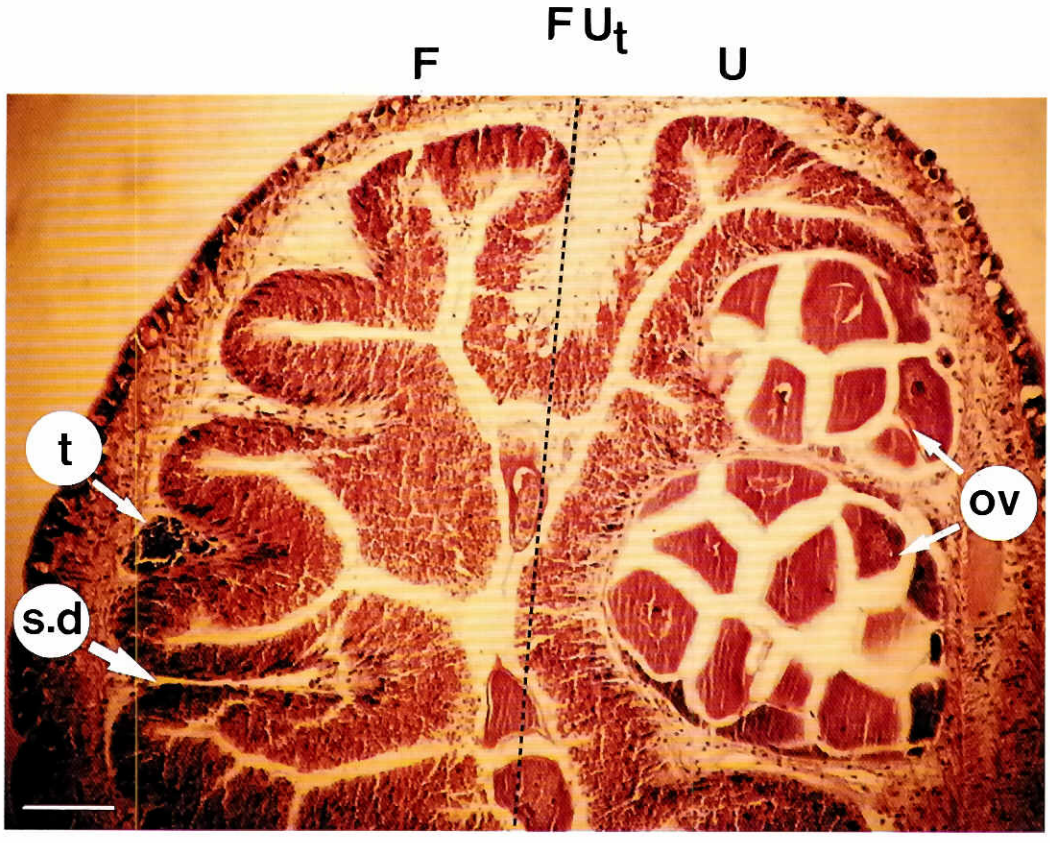

Fig. 7. Transection of an allophenic worm from the subclone FU_t, showing the asymmetric gonadogenesis in the 2 halves. *The initially male half F (left) contains sterile diverticles (s.d) except in one of them where a young testis (t) differentiated. In the initially female half U (right) bulky ovaries (ov) developed. Bar, 100 μm.*

Materials and Methods

Cloning of normal and chimeric worms

These studies were carried out on *L. sanguineus* stem worms from 4 clones (C_4, U_1, FU and UF) maintained in the laboratory. The first clone (C_4) was obtained by asexual multiplication of a male *L. sanguineus* specimen collected on the Brittany coast of the English Channel. Adult C_4 worms are dark-brown. The second clone (U_1) was produced by asexual reproduction of a female *L. sanguineus* collected on the Uruguayan shores of the South Atlantic Ocean. This female was selected because of its light-brown pigmentation and its normal oocyte development which contrasts with the abortive oogenesis of *L. sanguineus* females from the Brittany coasts of the Channel (Gontcharoff, 1951; Bierne, 1970).

Two animals of opposite sexes were sagitally cut in two. The female lateral halves were grafted onto the anatomically complementary male halves so as to construct 2 symmetrical heterosexual chimeras, FU (male/female) and UF (female/male) (Fig. 1) (Sivaradjam and Bierne, 1980). The original FU and UF chimeras grew into large chimeras. These chimeras were divided transversally into many pieces to produce the 2 clones – FU and UF – of bilaterally allophenic animals by both anterior and posterior regeneration (somatic embryogenesis) from fragments (Fig. 2).

Normal and chimeric worms were reared under standard laboratory conditions for nemertines: constant temperature (12°C), continuous darkness and food (calf liver) once a week.

Sample preparation

Biochemical studies were done on extracts of fasting animals. Fragments, i.e. bisected pieces or either right or left halves, of the gonad-containing module (segment-like in non-segmented worms) were cut with a surgical scalpel. The gonad-containing module is the 8th in the anterior-posterior body plan (Bierne, 1985). Eggs or sperm were collected in sea water after natural emission, or isolated by pressing the mature fragments between slides.

Fragments with gonads, isolated eggs or sperm, were placed in tubes containing 1 vol cold 10 mM Tris-HCl, pH 7.4 and sonicated. One volume of lysis buffer (2% Chaps, 2% DTT, 10% SDS) was added to each sonicated preparation, which was then shaken and heated at 100°C for 5 min. The same volume of a second lysis buffer (9.5 M urea, 5% DTT, 2% Chaps) was added and the samples were centrifuged at 12,000g for 5 min. The soluble proteins in the supernatant were carefully collected and stored as aliquots at -20°C.

The protein concentrations of the samples were determined by the method of Lowry *et al.* (1951). Proteins were precipitated from the supernatant with 2 vol of ice-cold acetone, centrifuged at 12,000g for 5 min, washed with ethanol and finally dried under vacuum.

Polyacrylamide gel electrophoresis

Protein samples prepared as described above were suspended in 30 μl sample-loading buffer (62.5 mM Tris-HCl, pH 6.8, 5% ß-mercaptoethanol, 10% glycerol, 3% SDS, 0.01% bromophenol blue), and heated at 100°C for 5 min, and analyzed by electrophoresis in polyacrylamide (6-15% linear gradient) slab gels using the discontinuous buffer system of Laemmli (1970). The proteins (40 μg per lane) resolved by this method (SDS-PAGE) were visualized by staining for 1 h in 0.1% Coomassie blue in acetic acid:methanol:H2O (1:5:4 by vol) followed by destaining in the same solution without the dye. Protein-relative molecular masses were estimated by reference to the migration of a set of "low" and "high molecular weight" marker proteins (Bio-Rad Laboratories, Richmond, USA) from 14,400 to 200,000 Daltons.

Histological staining

Worm fragments were fixed in Bouin's solution, embedded in paraffin and sectioned. The sections (6 μm) were stained with hematoxylin and eosin by standard procedures.

Acknowledgments

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