MONOGENIC REPRODUCTION ALLOWS COMPARISON OF PROTEIN PATTERNS OF FEMALE AND MALE PREDETERMINED OVARIES AND EMBRYOS IN CHRYSOMYA RUFIFACES (DIPTERA, CALLIPHORIDAE)

CHRISTIANE KIRCHHOFF* and VOLKER SCHROEREN
Zoologisches Institut der Universität, Biologiezentrum, Olshausenstr. 40-60, D-2300 Kiel, FRG

(Received 24 February 1986)

Abstract—1. Females of the monogenic blowfly Chrysomya rufifacies produce either female or male progeny depending on their genetic constitution, i.e. the presence or absence of a germ-line autonomous maternal effect sex realizer.

2. This type of sex determination allows a comparison of female and male predetermined ovaries and embryos on the molecular level.

3. The patterns of steady-state and newly synthesized proteins were analyzed as well as of cell-free translation products of poly(A)+RNA from ovaries and eggs of opposite future sex.

4. The results show that a small transient or quantitative difference in protein synthesis can be taken as a molecular indicator for female vs male development.

INTRODUCTION

Sex determination is the initial process that creates the morphological, physiological and behavioural differences between the sexes by switching on one of the two alternative developmental programs "female" and "male". In Drosophila melanogaster, as in other organisms whose genetics of sex determination have been studied, it is achieved be a control system in which a chromosomal signal affects a small number of regulatory genes (Baker and Belote, 1983).

However, sex determination in D. melanogaster does not solely depend on the X:A ratio of the zygote but rather on a relationship between the zygotic chromosome complement and some maternally specified constituents of the egg that are synthesized during oogenesis (Cline, 1983). The maternal components are necessary prerequisites for sex determination, but since they are present in equal amounts in all eggs of a wild-type female they have no discriminative power on their own. Thus, the genotype of the zygote remains the only discriminator in D. melanogaster.

In contrast, in the blowfly Chrysomya rufifacies sex of the progeny depends only on the maternal genotype with respect to a sex realizer gene F' (Ullerich, 1973). F'/f mothers are female producing (thelygenic or t-type females), f/f mothers are male producing (arrhenogenic or a-type females). This phenomenon is called "monogenic reproduction". The maternal effect of the female sex realizer F' indicates that its function, possibly carried on by a maternal gene product, must be present in the oocytes of t-type females before the end of meiosis (Ullerich, 1973).

The F' function is germ-line autonomous in females (Ullerich, 1984). However, when transplanted into a male embryo, F' bearing pole cells differentiate into functional sperms in the adult male. The male sex of the resulting F'/f progeny after mating of the germ-line mosaic males with a-type females indicates that the F' function was not exerted during spermatogenesis or embryogenesis to direct differentiation into the female sex (Ullerich, 1984). These data strongly suggest that F' function is restricted to female germ-line cells leaving oocytes and/or nurse cells of the meristic ovary as sources for a hypothetical "feminizing" F' product.

The genetic basis of monogenic reproduction and the maternal effect of the sex realizer allow separation of sex predetermined ovaries and embryos. This makes Ch. rufifacies a favorable object for studies of sex determination in insects on the molecular level. In this study it was intended to find sexual dimorphisms of protein patterns during developmental stages that do not yet display morphological dimorphisms between the sexes. For this purpose, the patterns of tissue proteins and of cell-free translation products of poly(A)+RNA from ovaries and eggs of known future sex were compared by one- and two-dimensional gel electrophoresis.

The results presented here clearly show that no major sex-linked differences in the protein patterns preceded the morphological differentiation of the sexes. Only a minor translation product was identified displaying a reproducible pattern of sex-linked synthesis, possibly representing differential gene expression. First results of these experiments have been published recently by Kirchhoff (1986).

*Author to whom correspondence should be addressed at: Department of Genetics, Katholieke Universiteit, Toernooiveld, 6525 ED Nijmegen, The Netherlands.
MATERIALS AND METHODS

Collection of ovaries and embryos of known predetermined sex

A wild-type laboratory stock of Chrysomya rufifacies (Diptera, Calliphoridae) was maintained as described by Ullerich (1973). Ovary follicles in a female develop strictly synchronously, the growth of the younger follicle generation being repressed until the terminal oocytes are deposited. One female deposits clusters of about 100 eggs which also develop synchronously. Eggs from isolated females were collected over a period of max. 30 min, dechorionized in 20% chloroform and frozen in liquid nitrogen after they had reached the blastoderm stage (2.5-3 hr after deposition, 22 °C incubation temp.). Part of the eggs was allowed to develop into adult flies for sex control. The second follicle generation of the isolated females was dissected after 2-3 days, staged by morphological criteria according to Bier (1963), frozen in liquid nitrogen and stored at −70 °C until the control flies eclosed. The oogenesis stages laid down for the cycloraphic flies roughly correspond to the following stages of Drosophila melanogaster ovaries: stage 3 of Ch. rufifacies corresponds to stages 8-9, stage 4 corresponds to stages 10-11, stage 5 corresponds to stages 12-13 and stage 5 to stage 14 oocytes. Ovaries and eggs of at least five isolated females were incubated according to female and male sex for every sample preparation, in order to minimize stage inhomogenities of different samples which could not be ruled out completely by inspection of ovaries and embryos.

Labeling of ovaries and embryos in vivo

Approximately 10 μCi of 35S-methionine (Amersham, translation grade, ca. 1000 Ci/mMol) was injected into the abdomen of a female 1 hr before the dissection of the second generation of ovary follicles. Embryos were labelled during blastoderm formation using the technique of Limbourg and Zalokar (1973). Egg clusters of isolated females were incubated in a wet chamber after chemical dechorionization. Their development was observed until they reached the stage of pole cell formation (2-2.5 hr after deposition). At this time the eggs were permeabilized using octane. Embryos (50-80) were incubated for 1 hr in 50 μl sterile medium (70 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM H₂O, 2 mM Na₂CO₃) containing 100 μCi of 35S-methionine (Amersham). After the incubation time, embryos were washed in cold medium and frozen in liquid nitrogen.

Preparation of poly(A)+RNA of ovaries and eggs

Total RNA was extracted from staged ovaries and embryos that had been pooled according to their predetermined sex as described by Kirchhoff (1981). Poly(A)+RNA was isolated by oligo(dT)-cellulose chromatography as described earlier (Kirchhoff, 1986). The yields of RNA were estimated by optical density measurements at 260 nm. Poly(A)+RNA fractions were precipitated by ethanol, redissolved in sterilized H₂O bidest at a final concentration of 1 μg/μl, subdivided and stored at −70 °C. The molecular weight distribution of every poly(A)+RNA preparation was tested by electrophoretic separation of 5 μl aliquots on 1.5% agarose gels under denaturing conditions according to Brandt and Hackett (1983) and visualized by ethidium bromide staining.

Cell-free translation of poly(A)+RNA

Poly(A)+RNA of ovaries and embryos was translated in both the rabbit reticulocyte lysate system (Amersham, nu-clease treated product) and the wheat germ system (nuclease treated, kindly provided by Professor Dr K. Apel). 35S-methionine (Amersham, translation grade, sp. act. ca. 1000 Ci/mMol) was applied at a final concentration of 1 μCi/ml. The final concentration of poly(A)+RNA was 0.1 μg/ml. The reticulocyte lysate translation mixtures were incubated for 90 min at 30 °C, the wheat germ translation mixtures were incubated for 90 min at 25 °C. Reactions were stopped adding 25 vol % of 100 mM EDTA and 300 μg/ml RNase A and a second incubation at 37 °C for 15 min. The incorporation of 35S-methionine into TCA-insoluble material was assayed from 2 μl aliquots as described by O’Farrell (1975).

Protein sample preparation

For one-dimensional SDS-PAGE (see below) cell-free translation products and tissue proteins were adjusted to a final concentration of 3% SDS, 0.06 M Tris-Cl (pH 6.5), 10% (v/v) β-mercaptoethanol and boiled for 1 min. For two-dimensional analysis (see below) protein samples were brought to lysis buffer concentration according to O’Farrell (1975). To minimize proteolysis, tissue samples were previously sonicated or homogenized in 0.1% SDS, 0.01 M Tris-Cl and boiled for 10 min. About 100 μl SDS-solution were used per 10 mg of tissue. Afterwards either the whole samples were brought to lysis buffer concentration or it was centrifuged for 10 min at 12,000 g to separate the SDS-insoluble proteins from the insoluble bulk of proteins containing most of the yolk proteins. The SDS-insoluble proteins were dissolved in 9.5 M urea and referred to as the unsoluble protein fraction.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were subjected to SDS-PAGE according to Laemmli (1970) with the following modifications. The stacking gels were polymerized adding 0.01 M riboflavin. The separating gels were mixed with a linear gradient from 10 to 20% acrylamide. Unlabelled proteins were separated on 120 × 70 × 2.7 mm gels and stained with 0.025% Coomassie blue and/or silver stain (Oakley et al., 1980). Radioactive samples were separated on 180 × 180 × 1.5 mm gels and fluorographed as described by Bonner and Laskey (1974) using Kodak X-AR 5 autoradiography film. Between 30,000 and 100,000 cpm of 35S-labelled proteins were loaded per gel slot.

Two-dimensional gel electrophoresis (2-DE)

The two-dimensional techniques described by O’Farrell (1975) and O’Farrell et al. (1979) were employed with the following modifications. The focusing gels contained amphotoline mixtures of two parts Servalytes, pH 5-7, and 1 part Ampholytes, pH 3.5-10. Isoelectric focusing (IEF) was performed at 10,000 V hr (Vh). Non-equilibrium pH gradient electrophoresis (NEPHGE) was performed at 2000 Vh. Focusing gels were frozen in equilibration buffer (O’Farrell, 1975) and stored at −70 °C. In order to determine the pH-gradient, a focusing gel was frozen directly after IEF or NEPHGE and sliced into pieces of equal size. Each piece was shaken in 1.0 ml degassed distilled water for 1 hr and the pH of the solution measured. In some experiments proteins of known isoelectric points (commercial products) were used as pl markers. Second dimensions were run according to Laemmli (1970) in the case of unlabelled proteins (see above). Radioactive samples were run on 180 × 180 × 1.5 mm gels using a linear gradient from 7.5 to 15% acrylamide. Approximately 200,000 cpm of 35S-labelled proteins were loaded per focusing gel. Exposure times were about 10 days. Protein patterns were analyzed by inspection. To determine the molecular weight distribution a mixture of mol. wt marker proteins (Pharmacia) was included in the agarose layer of the 2-DE gels. Marker proteins were made visible by staining with 0.025% Coomassie blue.

RESULTS

Protein patterns of t-type and a-type ovaries and embryos

The patterns of steady state proteins of both ovaries and embryos spanning a period from the
Fig. 1. SDS-PAGE protein patterns of a-type and t-type ovaries and blastoderm embryos of opposite sex. Even numbered lanes contain t-type or female proteins. Comparable amounts of protein prepared from 5–10 ovary follicles or dechorionized eggs were loaded per gel slot. Urea-soluble and SDS-soluble protein samples were prepared as described in Materials and Methods, separated on 10–20% LAEMMLI-gradient gels and stained with Coomassie blue. Approximate mol. wts are given in kilo Daltons (kd). Protein bands showing stage-specific differences are marked (*). Lanes 1–3: stage 3 ovaries, lane 1 contains urea-soluble, lanes 2 + 3 SDS-soluble proteins. Lanes 4–7: stage 5 ovaries, lanes 4 + 5 contain urea-soluble, lanes 6 + 7 SDS-soluble proteins. Lanes 8–11: stage 6 ovaries, lanes 8 + 9 contain urea-soluble, lanes 10 × 11 SDS-soluble proteins. Lanes 12–15: blastoderm embryos, lanes 12 + 13 contain urea-soluble, lanes 14 + 15 SDS-soluble proteins. Putative yolk proteins are designated (YP 1-3).
Fig. 2. 2-DE protein patterns of t-type and a-type ovaries of different oogenesis stages. Total protein samples of about 20-50 follicles were loaded per NIEPHGE-gel. Second dimensions were on 10-20% LAEMMLI-gradient gels; proteins were visualized using silver stain. Given pH values were estimated from reference gels. Horizontal lines mark the position of mol. wt markers as described in Materials and Methods. (a) total proteins of t-type ovaries of stage 4 of oogenesis; (b) total proteins of a-type ovaries of the same stage; (c) total proteins of t-type ovaries of stage 6; and (d) same of a-type ovaries. Putative yolk proteins (YP 1-3), actins (A) and tubulines (T) are designated. The sex-linked difference apparent in Fig. 5 was not observed in these gels due to the poor resolution of tissue proteins.

Differences between the newly synthesized proteins of t-type and a-type ovaries were not observed at either oogenesis stage (data not shown).

Fig. 3. Fluorographs of newly synthesized ovary proteins (a) and cell-free translation products of total poly(A)+RNA (b) of different oogenesis stages (not separated according to predetermined sex). Stages are indicated above each track. Ten to twenty-percent of PAA LAEMMLI-gradient gels containing mol. wt markers were stained, dried and exposed to X-ray film. Approximately 30,000 cpm of in vivo 35S-labelled ovary proteins (see Materials and Methods) were loaded per gel slot. Exposure time was 5 days. Putative yolk proteins are designated (YP 1-3). (b) Approximately 100,000 cpm of 35S-labelled reticulocyte lysate translation mixture (see Materials and Methods) were loaded per gel slot. Exposure time was 24 hr. A main band comigrated with actin from rabbit muscle (A); a weak background band was observed on minus poly(A)+RNA tracks between 40,000 and 50,000 (not shown).

During the blastoderm stage, permeabilized eggs of Ch. rufifacies incorporated an average of about 25,000 cpm of 3S-methionine into TCA-insoluble material. Nearly all of the radioactivity was found in the SDS-soluble protein fraction. The small residual radioactivity of the urea-soluble fraction was not analyzed. Since the bulk of the unlabelled yolk proteins was excluded from the SDS-soluble fractions, the resolving power of the 2-DE technique was enhanced compared with the separations of the ovary proteins [Fig. 4(a), (b)]. Furthermore, the specific activities were higher. Thus, the overloading effects as well as severe SDS effects were prevented. The 2-DE separations of newly synthesized blastoderm proteins resulted in a total of about 500 polypeptide spots. Again, however, sex-linked differences were not observed.

Cell-free translation of poly(A)+RNA of ovaries and oocytes

Total poly(A)+RNA prepared from ovaries of t-type and a-type females at different stages of oogenesis was translated in both the reticulocyte lysate and the wheat germ system. Accordingly to their molecular weight distribution during SDS-PAGE, the cell-free translation products of the reticulocyte lysate system resembled very much the proteins labelled in vivo, although remarkable quantitative differences occurred [Fig. 3(a), (b)]. Most of the polypeptide coding poly(A)+RNAs seemed to be present during the whole investigated period of oogenesis [Fig. 3(b)] and early embryogenesis (data not shown). A polypeptide band comigrating with the actin marker was one of the most prominent translation products at all examined stages. Most of the stage-specific differences in the protein patterns were only of a quantitative character.

The most prominent difference between the ovary proteins labelled in vivo [Fig. 3(a)] and the cell-free translation products of ovarian poly(A)+RNA [Fig. 3(b)] concerned the putative yolk proteins. Although translation products resembling the yolk proteins...
Protein patterns of female and male predetermined ovaries and embryos

Fig. 4. Fluorographs of 2-DE separations of newly synthesized proteins of female (a) and male (b) blastoderm embryos. Approximately 200,000 cpm SDS-soluble proteins of about 10 in vitro \(^{35}\)S-labelled embryos (see Materials and Methods) were loaded per IEF-gel. Second dimensions were on 7.5 to 15% NEVILLE-gradient gels. Gels were dried and exposed to X-ray film for 14 days. Given pH gradient and mol. wts were estimated from reference gels. (a) Proteins of female blastoderm embryos; (b) proteins of male blastoderm embryos. Sex-linked differences were not observed.

were observed at all stages of oogenesis of Ch. rufifacies, their amount did not exceed the other major translation products. Therefore, together with the high specific activities of the translation mixtures (at least 10 times higher than in the in vivo labelled proteins), the cell-free translation products met the requirements for high resolution 2-DE.

2-DE patterns of cell-free translation products of ovarian poly(A') RNA

The 2-DE fluorograms of the poly(A') RNA translation products showed roughly 800 spots in a highly reproducible pattern (Fig. 5). Combining IEF (O'Farrell, 1975) and NEPHGE (O'Farrell et al., 1979) a minimum of 900 different polypeptides was found consistently. The major translation products of all examined stages of development were considered as actin and tubulin products by coelectrophoresis with actin and tubulin marker proteins. Yolk protein-like translation products were not identified with certainty on 2-DE fluorograms since there were many polypeptides running in the corresponding area of the gels (Kirchhoff, 1986).

Our results indicated that most of the major polypeptide coding poly(A') RNAs were always present in both t-type and a-type ovaries. Out of the total number only 16 spots displayed a reproducible pattern of stage-specific synthesis. This small number was in the range of the stage-specific differences observed in other systems (Gutzeit and Gehring, 1979; Ingman-Baker and Candido, 1980; Jäckle and Kalthoff, 1980; Savoini et al., 1981; Greenberg and Adler, 1982).

However, one acidic polypeptide (pI about pH 4.2) of approx. 25,000 of t-type ovaries was left undetected in the patterns of vitellogenic a-type ovaries [stages 3 and 4, Fig. 5(b)]. Since this polypeptide was observed in both types of stage 5 ovaries and mature oocytes (stage 6), the only sex-linked difference observed in our analysis was transient rather than permanent. An alternative interpretation from our fluorograms is a small quantitative difference in its overall level.

DISCUSSION

In D. melanogaster there is indirect evidence that sex determination occurs around the blastoderm stage (Baker and Belote, 1983). The state of sex determination seems to pervade every single blastoderm cell and its descendants. This led to the assumption that developmental stages and tissues displaying no overt sexual dimorphism nevertheless may have sex in terms of differential gene expression in females and males (Nöthiger and Steinmann-Zwicky, 1985), this possibly being reflected by differential protein patterns.

In the monogenic blowfly Ch. rufifacies there seemed to be a direct way to test this assumption, since female and male predetermined ovaries, oocytes and embryos can be separated by a straightforward method because of the characteristic mechanism of sex determination. On the level of polypeptide synthesis the methods of SDS–PAGE and even more of two-dimensional electrophoresis (2-DE) allow to examine changes in gene expression in great detail. However, our results show that most of the major proteins are always present and always synthesized. A maximum of about 800 gene products could be analyzed from a single 2-DE fluorogram. Long-time exposure of the fluorograms revealed only about 100 additional spots. It therefore appears that the frequency distribution of proteins decreases significantly beyond these 800 most abundant proteins and that many proteins which might be modulated during developmental processes are present in quantities far
Fig. 5. Cell-free translation products of poly(A)$^+$ RNA of t-type (a + c) and a-type (b + d) ovaries of different developmental stages. Approximately 200,000 cpm of $^{35}$S-labelled reticulocyte lysate translation mixture (see Materials and Methods) were loaded per IEF-gel. Second dimensions were on 7.5–15% NEVILLE-gradient gels. Gels were dried and fluorographed for 10 days. Molecular weights (in kd) were estimated from reference gels. Only the acidic and low molecular weight products are shown in the fluorograms. (a) cell-free translation products of poly(A)$^+$ RNA of t-type ovaries of stage 4 of oogenesis; (b) same of a-type ovaries; (c) cell-free translation products of t-type ovaries of stage 6; and (d) same of a-type ovaries. The polypeptide displaying a sex-linked synthesis pattern is encircled (O).

below the level of detection even of 2-DE gels. As a consequence t-type and a-type ovaries and oocytes as well as blastoderm embryos of opposite sex which appear identical at the morphological level could not be distinguished regarding their most abundant proteins (Figs 1–4). Obviously even a fundamental difference such as the future sex is not preceded and therefore cannot be caused by major differences in the protein patterns. Rather, the biochemical basis of sex determination seems to be very subtle in nature in Ch. rufifacies.

Maximum resolving power was reached at the level of cell-free translation products (Fig. 5). A single acidic 25,000 polypeptide showed a different synthesis pattern in t-type and a-type ovaries. However, from our experiments we cannot decide whether this difference is correlated causally with the state of sex determination. There are examples that region-specific differences in protein patterns which are correlated with spatial organization, are transient, too (Gutzeit and Gehring, 1979; Jäckle and Kalthoff, 1980, 1981; Greenberg and Adler, 1982).

Ullerich (1973) has proposed a model of F' functioning in Ch. rufifacies ovaries. A female determining F' gene product is supposed to be synthesized during the oogenesis of t-type females. Evidently, this gene product should at no time be found in ovaries of a-type females. The only sex-linked difference observed in our analysis does not match this model (Fig. 5) and most probably does not refer to the F' gene.

Nevertheless, our results show that there are differences between male and female predetermined ovaries at least during certain stages of oogenesis. The sex of a fly which would have developed, after fertilization, could be predicted from the 2-DE pat-
terns of cell-free translation products of poly(A)⁺ RNA.

Acknowledgements—The authors would like to thank M. Martensen and K. Bielenberg for helping with the breeding of the flies and L. Riepen for helping with the photographs. Dr H. Jäckle, Prof. Dr F.-H. Ullerich and Dr H. Zacharias are thanked for helpful discussions and critical reading of the manuscript. Certain modifications in the two-dimensional gel electrophoresis were based on suggestions of I. Häuser and Prof. Dr K. Apel.

REFERENCES


