EXTRA-CHROMOSOMAL DNA IN EARLY STAGES OF OOGENESIS IN ACHETA DOMESTICUS

M. D. CAVE AND E. R. ALLEN
Department of Anatomy and Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A.

SUMMARY
A large extra-chromosomal DNA body is found in gonial and oocyte nuclei of Acheta domesticus. Somatic cells within the ovary do not contain the DNA body which is limited to the nuclei of gametogenic cells. During early prophase of meiosis this body is spherically shaped and intensely Feulgen positive. Electron microscopy shows it to be composed of tightly packed fibrogranular material. The body is formed in the nuclei of premeiotic interphase cells where it first appears as a mass of dense chromatin material located within the nucleolus. In nuclei of early prophase cells the body is closely associated with the nuclear membrane. It increases in size, reaching a maximum in mid-pachytene nuclei. During late pachytene-early diplotype stage of meiosis the tight fibrillar material within the body loosens and takes on a less compact appearance. At the same time large fascicles of RNA-containing material accumulate within and around the DNA body. The amount of RNA material surrounding the body increases as the oocytes proceed into an arrested diplotype stage of development.

INTRODUCTION
The concept that the size, number, and structural characteristics of chromosomes as well as the amount of DNA per haploid chromosome complement is constant within the cells of a given species of organism is a sine qua non in explaining some important aspects of genetics and differentiation. Notable exceptions to this rule are found in incidences of chromosome diminution, polyploidy and polyteny, and in cases of chromosome loss or rearrangement.

Extra-chromosomal DNA-containing bodies are found during oogenesis in several insects. Giardina (1901) described such a body in Dytiscus (Coleoptera). A similar body is found in the oocytes of Tipula (Diptera) (Bayreuther, 1952). Extra-chromosomal DNA is also found within the oocyte nuclei of amphibians (Painter & Taylor, 1942). Recent investigations show that this DNA is associated with peripheral nucleoli (Peacock, 1965; Miller, 1966).

The present study deals with a large DNA body which is present in oocytes of the house cricket, Acheta domesticus (Orthoptera). That this body is distinct from and present in addition to the chromosomes was shown by Nilsson (1966). The DNA body is first apparent in the nuclei of premeiotic interphase cells and is present throughout early prophase. Numerous RNA-containing structures develop in close association with the body. This paper deals with the morphological and histochemical changes which characterize this body as the cells proceed through the early stages of oogenesis.

In another paper (Allen & Cave, 1968), dealing with later stages of oocyte development, evidence is presented to show that the RNA-containing material which surrounds the DNA body represents ribosomal precursor material. Nucleocytoplasmic migration of this RNA-containing material is indicated.

MATERIALS AND METHODS

Female larvae of *Acheta domesticus* which were in the third to the fifth instar stages of development were used in these studies. The animals measured 7–13 mm in body length and weighed between 150 and 200 mg. The animals were anaesthetized with CO₂ and dissected in a small Petri dish.

For light microscopy the ovaries were immediately fixed in 3 parts ethanol: 1 part acetic acid or in phosphate-buffered 4% formaldehyde (pH 7.2). Ovaries fixed in formaldehyde were washed overnight in running water. The fixed ovaries were dehydrated and embedded in paraffin. Sections were cut at 5 μ and mounted on glass slides. After rehydration, the slides were stained according to the Feulgen procedure or with azure B according to the modification of Gabrusewycz-Garcia & Kleinfeld (1966). Sections of ethanol-acetic acid fixed ovaries were used in enzyme digestion experiments. For extraction of DNA, the slides were incubated at 37 °C for 3 h in 0.2% deoxyribonuclease (Worthington Biochemical Corp., Freehold, N. J.) prepared in 10⁻³ M MgSO₄ and adjusted to pH 7.0 with Na₂HPO₄. Ribonuclease digestion was accomplished by incubation of the preparations for 3 h at 37 °C in 0.03% ribonuclease (Worthington) adjusted to pH 7.0 with Na₂HPO₄. Following extraction the preparations were briefly rinsed in distilled water, washed for 10 min in 5% trichloroacetic acid (4 °C), and passed through three changes of 70% ethanol (10 min each). The specificities of the enzymic extractions were controlled by incubating sectioned preparations in identical solutions which did not contain the enzyme.

Light-microscope measurements were made with an eyepiece micrometer at a total magnification of 1500 x. Each figure represents measurements made on at least 100 cells.

For electron microscopy the ovaries were fixed for 3 h in 5% glutaraldehyde buffered to pH 7.2 with 0.1 M phosphate buffer. They were washed and stored overnight in fresh buffer solution. Subsequently the ovaries were post-fixed for 2 h in 1% osmium tetroxide in phosphate buffer. The ovaries were teased apart in the osmium solution. After washing, the specimens were stored for 24 h in fresh buffer solution. All fixations and washings were carried out at 0–4 °C. The specimens were dehydrated in an ethanol series and embedded in Epon 812 (Luft, 1961). Individual ovarioles were flat embedded in Petri dishes and oriented in such a way as to enable recognition of the terminal filament and oviduct ends of the ovariole. Alternate thick and thin sections were cut with glass knives on a Huxley ultramicrotome. Thick sections were mounted on glass slides and stained with aceto-orcein. These thick preparations served to identify the various stages of oocyte development present in a given region of the ovariole, before obtaining adjacent thin ones.

The thin sections were mounted on Formvar-coated, 200 mesh, copper grids and
DNA in oocytes of Acheta

stained with 1% aqueous uranyl acetate for 15 min followed by a 3-min exposure to 0.02% lead citrate. The grids were viewed in a Philips EM 200 electron microscope.

RESULTS

In the panoistic ovary of Acheta, the gametogenic cells in each ovariole are arranged in developmental sequence (Fig. 4). At the proximal end of the ovary, adjacent to the terminal filament, are found oogonia or premeiotic interphase cells. Oocytes in early prophase stages of meiosis follow in a sequential manner. The greatly enlarged cells at the distal end of the ovariole are in an arrested diplotene stage of meiotic prophase I.

In addition to the chromosomes, the nuclei of gametogenic cells contain a spherically shaped mass of Feulgen positive material (Figs. 3, 5, 6, 11-14, 15, 17, 20). That this intensely stained mass contains DNA is indicated by the fact that the Feulgen staining is absent in sections previously extracted with deoxyribonuclease (but not in sections treated with buffer solution in the absence of enzyme).

The chromosomes and DNA-containing body of early prophase cells stain orthochromatically (blue green) with azure B at pH 4.0. The cytoplasm stains metachromatically (purple). In the enlarged nuclei of diplotene cells, an accumulation of metachromatically stained material surrounds the DNA body (Fig. 21). Ribonuclease digestion followed by azure B staining results in a complete absence of the purple stain while the blue-green staining of the body and the chromosomes is retained. The purple stain is not removed from sections treated with RNase buffer prior to azure B staining.

Electron-micrograph profiles of premeiotic interphase cells display a large nucleus surrounded by a thin rim of cytoplasm (Fig. 2). Dense chromatin material is concentrated at the periphery of the nucleus. A large prominent nucleolus is apparent. Material morphologically identical to dense chromatin is localized in the centre of the nucleolus.

The distribution of Feulgen stain closely parallels the distribution of dense and diffuse chromatin (Fig. 3). Intensely stained accumulations of Feulgen positive material line the nuclear periphery. A mass of Feulgen positive material is also found in the centre of the nucleus. The size of this mass and its location indicate that it corresponds to the intranucleolar structure seen in the electron micrograph. A second Feulgen positive body, probably a sex chromosome, is present in some nuclei.

The Feulgen positive body is found only in the nuclei of germ line cells and not within the nuclei of follicle cells. Electron-microscope profiles of follicle cells display a nucleolus of uniform density (Fig. 1).

During early prophase, the Feulgen positive body lies close to the nuclear membrane. In zygotene stage nuclei the body is oval in shape and has an average diameter of 2.6 μ (Figs. 5, 6). In electron micrographs of zygotene cells the chromosomes are elongate masses of dense material which usually appear as paired elements (Fig. 7). The DNA body occupies a large portion of the nucleus. Large spaces, possibly vacuoles, may be found within the body at this time. Associated with the body are small fascicles of electron-dense material. These fascicles are morphologically identical to the RNA-containing material found during later stages of oocyte development.
The chromosomes of pachytene stage nuclei are intimately paired. During mid-pachytene the chromosomes condense and move towards one end of the nucleus while the Feulgen positive body locates at the opposite end (Figs. 11-14). The average diameter of the DNA body measures 3.2 μ in mid-pachytene nuclei. The chromosomes are distinguished by axial core structures (Figs. 8, 9). The tripartite nature of these elements can be readily distinguished at higher magnifications (Fig. 10). Axial cores were not visible during earlier or later stages of oogenesis. Except for an increase in size, the morphology of the body during mid-pachytene does not differ from that observed in zygotene nuclei.

During late pachytene, the chromosomes become increasingly diffuse and lose their sharp stainability. The Feulgen positive body undergoes a similar change in appearance (Figs. 14, 15, 17). Feulgen positive material around the periphery of the body stains very weakly (Fig. 15). These cytochemically detectable changes in the body can be explained by modifications of ultrastructure. In electron micrographs the body is no longer a dense mass but presents a less compact appearance (Fig. 16). Electron-dense (RNA containing) material surrounds the diffuse DNA body at this time.

In late pachytene-early diplotene nuclei the chromosomes are very diffuse and can no longer be demonstrated by ordinary cytochemical procedures (Fig. 17). The Feulgen positive body is closely apposed to the nuclear membrane. Distinct chromosome structures are no longer visible in electron micrographs (Fig. 18). The DNA body becomes more dispersed, and large amounts of electron-dense material accumulate within it.

During diplotene stage of prophase I, the nucleus increases in size. The chromosomal material remains diffuse (Fig. 20). The DNA body is located near the centre of the nucleus and is surrounded by a large accumulation of material which stains purple with azure B (Fig. 21). Electron micrographs show that the body is filled with fibro-granular material which appears to be arranged in the form of bundles (Fig. 19). The body is surrounded by a large accumulation of electron-dense material which displays a highly organized structure.

**DISCUSSION**

Extra-chromosomal DNA has been observed in germ line nuclei of various organisms. In the meristic ovary of *Dytiscus marginalis* an extra-chromosomal DNA body appears during oogonial proliferation (Giardina, 1901). An oogonium containing the body divides and the body is included in one of the daughter cell nuclei. Three subsequent divisions occur to form a total of 16 cells. The DNA body is passed on to only one of these cells, the oocyte. The remaining 15 cells which lack the body become nurse cells.

A similar DNA body is found within the oocyte nuclei of *Tipula* (Bauer, 1931; Bayreuther, 1956). The body is replicated once during oogonial mitoses so that it is found in one of the 15 nurse cells as well as in the oocyte.

In oocytes of *Tipula oleracea* the DNA body is a sphere measuring up to 6 μ in diameter (Lima-de-Faria, 1962). Within the body are found one or more nucleoli.
each displaying the elaborate nucleolonema (Lima-de-Faria & Moses, 1966). The DNA bodies of both Acheta (this investigation) and Tipula are composed of tightly intertwined fibrillar material. Observations made upon diplotene nuclei of Acheta show that the body contains bundles of similarly aligned fibres. As in early prophase nuclei of Acheta, the DNA body of Tipula is closely associated with the nuclear membrane. Separating the body from the remainder of the nucleus in Tipula oocytes is an outer shell consisting of material rich in RNA. A clearly defined thin outer shell similar to that observed in Tipula was not observed in the present investigation.

In Dytiscus, Tipula, and Acheta the extra-chromosomal DNA body undergoes a similar life cycle. It first appears in the premeiotic interphase cells, increases in size during the early stages of prophase I, and is no longer demonstrable in late diplotene oocytes (Allen & Cave, 1968). In Tipula and in Acheta the DNA body is associated with RNA-containing material similar to that found in the nucleolus (Chouinard, 1966). Granules ranging between 110 and 150 Å in diameter and resembling cytoplasmic ribosomes are associated with the DNA body of Acheta (Allen & Cave, 1968). Small accumulations of RNA have been demonstrated histochemically within the DNA body of Dytiscus marginalis (Urbani & Russo-Caia, 1964). The ultrastructure of this material was not reported.

Germinal vesicles of amphibian oocytes may contain hundreds of extrachromosomal nucleoli (Gall, 1954; Callan, 1966). In somatic tissues of the same individuals the number of nucleoli corresponds to the number of nucleolar organizer regions, usually one per haploid set (Dearing, 1934). Increases in the number of nucleoli usually reflect an increase in ploidy and indeed estimates of ploidy have been based upon the number of nucleoli present in a cell (Fankhauser & Humphrey, 1943). This is not true for the germinal vesicle where in spite of tremendous increases in the number of nucleoli the basic chromosome number remains diploid (Callan, 1966). In addition to DNA found in the chromosomes, the germinal vesicle contains extra-chromosomal DNA (Painter & Taylor, 1942).

The extra nucleoli appear in the germinal vesicles of amphibians during the pachytene stage of meiotic division I (Callan, 1966; Gall, 1968). At the same time Feulgen positive granules appear in the nucleus. The appearance of these granules is correlated with a tremendous increase in the synthesis of DNA which codes for ribosomal RNA (Gall, 1968). Synthesis of this DNA is completed before the cells proceed into the diplotene stage of meiosis.

In Xenopus laevis and in Plectodon vehiculum each nucleolus contains a ring-shaped structure the core of which is composed of DNA (Miller, 1966; Peacock, 1965). These DNA rings are closely associated with the nuclear membrane. Miller (1966) suggests that this extra-chromosomal DNA in Xenopus has arisen by an amplification of the chromosomal nucleolar organizer region.

The relationship between the nucleolus and ribosomal RNA precursors is well established (Edström & Daneholt, 1967; Brown & Gurdon, 1964). DNA-RNA hybridization experiments show an increased amount of DNA homologous to ribosomal RNA in germinal vesicles of Xenopus laevis (Brown & Dawid, 1968). The amount of DNA homologous to ribosomal RNA in the oocyte nucleus is about 1500 times that
present in a somatic cell. Yet the total amount of DNA in germinal vesicles is no greater than 23 times the haploid DNA value. Comparisons between germinal vesicles and liver cells show that relative to the amount homologous to 4S RNA, germinal vesicles contain a much greater amount of DNA homologous to ribosomal RNA.

The DNA body of *Acheta* is first apparent within the nucleolus of oogonial cells. During diplotene stage the DNA body is surrounded by RNA-containing material in which are found granules similar in size and appearance to cytoplasmic ribosomes (Allen & Cave, 1968). The close association between the DNA body and this RNA suggests that the situation in *Acheta* is similar to that described in amphibians, the DNA in the body being formed by repeated replication of ribosomal cistrons within the nucleolar organizer region of the chromosomes. Preliminary cytological observations suggest that the body may be formed in close association with one of the autosomes (Nilsson, 1966).

Appreciable quantities of RNA surrounding the body are not found until diplotene stage. At this time RNA is synthesized on or accumulates around the DNA body. The role of the DNA body might be similar to that of the extra-chromosomal DNA found in the nucleoli of amphibian oocytes. In *Acheta* this DNA is organized into a particulate mass measuring up to 4.0 μ in diameter. Whether or not the DNA in the body is subsequently redistributed or broken down when the DNA body disappears is a question which presently occupies our attention.

The authors are indebted to Dr D. Ammermann, Zoologische Institut, Tübingen, W. Germany, for providing the *Acheta domestica* used in this study. We gratefully acknowledge the technical assistance of Miss Agnes Cralley.

This work was supported by grant number 7-F2CA-23, 971-01A1 from the United States Public Health Service and a grant from the Health Research Services Foundation (number 1-A1).

REFERENCES


DNA in oocytes of Acheta


(Received 22 July 1968)
Fig. 1. Typical follicle cell located at the periphery of an enlarged oocyte. $n$, nucleolus. $\times 10140$.

Figs. 2, 3. Premiotic interphase cells seen in the proximal region of an ovariole.

Fig. 2. Electron micrograph. The nucleus is distinguished by a large centrally located mass of dense chromatin material (DNA body). $n$, nucleolus. $\times 43000$.

Fig. 3. Feulgen stained preparation. An intensely Feulgen positive mass corresponds to the DNA body (long arrow). Another mass of Feulgen positive material, a presumptive sex chromosome, is also present (short arrow). $\times 3680$. 
DNA in oocytes of Acheta
Fig. 4. Feulgen stained section through the proximal portion of an ovariole. The sequential arrangement of cells in various stages of meiotic prophase I is demonstrated. The body is readily apparent in zygotene and pachytene cells (arrows). (d, early diplotene; p, pachytene; pm, premeiotic interphase; z, zygotene.) × 2000.

Figs. 5–7. Cells in zygotene stage of prophase I.

Figs. 5, 6. Feulgen stained preparations. DNA body is distinctly formed and is separate from the remainder of the chromosomes (b, DNA body). × 2520.

Fig. 7. Electron micrograph. Associated with the body are small amounts of electron-dense (RNA-containing) material. (b, DNA body; arrows, paired meiotic chromosomes.) × 6000.
DNA in oocytes of Acheta

Figure 4: [Image description]

Figure 5: [Image description]

Figure 6: [Image description]

Figure 7: [Image description]
Figs. 8–14. Cells in pachytene stage of prophase I.

Figs. 8, 9. Electron micrographs. A small amount of electron-dense (RNA-containing) material is associated with the DNA body. Nuclei of pachytene stage cells contain axial core structures (arrows). × 6340.

Fig. 10. Higher magnification of an area in the nucleus of the cell shown in Fig. 8. The micrograph reveals the typical tripartite structure of the axial core complex (arrows). × 54,000.

Figs. 11–14. Feulgen stained preparations of cells in early (Fig. 11), mid (Figs. 12, 13), and late pachytene stages (Fig. 14) of meiotic prophase I. During this time the chromosomes condense and become located at one end of the nucleus. Figs. 11–13, × 3600. Fig. 14, × 2500.
DNA in oocytes of Acheta
Figs. 15, 16. Cells in late pachytene stage of meiotic prophase I.

Fig. 15. Feulgen stained preparation. The chromosomes take on a diffuse appearance and begin to lose their sharp stainability. In a similar manner, the DNA at the periphery of the body becomes less condensed and gives the body an indistinct or fuzzy appearance. $\times 2400$.

Fig. 16. Electron micrograph. In late pachytene nuclei the DNA body presents a more irregular shape and appreciable amounts of electron-dense (RNA-containing) material begin to accumulate around it. $\times 5750$.

Figs. 17, 18. Cells in late pachytene-early diplotene stage of meiotic prophase I.

Fig. 17. Feulgen stained preparation. The DNA body lies very close to the nuclear membrane. The chromosomes, in a very diffuse condition at this time, are no longer demonstrated by ordinary cytochemical methods. $\times 2400$.

Fig. 18. Electron micrograph. The DNA body is no longer a compact mass of material but takes a very loosened or stretched appearance. Large amounts of electron-dense (RNA-containing) material are found distributed throughout the DNA body. $\times 5400$. 
DNA in oocytes of Acheta
Figs. 19–21. DNA body of cells in diplotene stage of meiotic prophase I.

Fig. 19. Electron micrograph. DNA body and associated electron-dense (RNA-containing) material from a diplotene cell. The electron-dense material is arranged in the form of discrete fascicles surrounding the DNA body. × 14,540.

Fig. 20. Feulgen stained preparation. The DNA body is located in the centre of the nucleus. The chromosomes are very diffuse and not distinguishable in ordinary sectioned material. × 2000.

Fig. 21. Azure B stained preparation. The DNA body and the nuclei of follicle cells stain orthochromatically (blue-green). The cytoplasm and material surrounding the body stain metachromatically (purple). × 2000.
DNA in oocytes of Acheta