INCORPORATION OF [^{3}H]URIDINE BY THE CHROMATOID BODY DURING RAT SPERMATOGENESIS

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ABSTRACT

The in vitro incorporation of tritiated uridine into RNA by the spermatogenic cells of the rat has been analyzed by high-resolution autoradiography. Special attention has been focused on the unique cytoplasmic organelle, the chromatoid body. After a short labeling time (2 h), this organelle remains unlabeled in the vast majority of the early spermatids although the nuclei are labeled. When the 2-h incubation with [^{3}H]uridine is followed by a 14-h chase, the chromatoid body is seen distinctly labeled in all spermatids during early spermiogenesis from step 1 to step 8. Very few grains are seen elsewhere in the cytoplasm of these cells. When RNA synthesis in the spermatid ceases, the chromatoid body also remains unlabeled. It is likely that the chromatoid body contains RNA which is synthesized in the nuclei of the spermatids. The function of this RNA as a stable messenger RNA needed for the regulation of late spermiogenesis is discussed.

The chromatoid body in the spermatogenic cells was first described by Benda (1) more than 80 yr ago. The questions of its origin, chemical composition, and function are, however, still unsolved. Light microscope histochemical techniques have suggested that it contains RNA and basic proteins rich in arginine (4, 27, 28), but electron microscope histochemical techniques have failed to confirm the presence of RNA in the chromatoid body (6). It has been suggested that the chromatoid body arises from the nucleus (27) or from the nucleolus (3). It also has been proposed that it has a cytoplasmic origin from the mitochondria (9). The changing relationships of this organelle during spermatogenesis, and the possible association with mitochondrial clusters during the pachytene stage and thereafter with the nuclear pores during early spermiogenesis, support the view that the chromatoid body may be dependent on both DNA-containing organelles of the cell (8).

The chromatoid body may have a role in the formation of the acrosomic system during early spermiogenesis and the flagellum and associated structures during late spermiogenesis, when RNA synthesis of the spermatids has ceased (9, 23). The observations which demonstrate that *(a)* the chromatoid body during early spermiogenesis has contact with intranuclear material through pores in the nuclear envelope, and *(b)* the intranuclear material appears to be similar in structure to the chromatoid body (30) have led us to assume that the chromatoid body has a role in the RNA metabolism of the developing male germ cells. This has now been studied further by using electron microscope autoradiography and labeling with tritiated uridine.

MATERIAL AND METHODS

Cell Separation and Labeling Conditions

Young adult rats (3–5 mo), derived from the Sprague-Dawley strain, were used in the experiments. They were

killed by a blow on the head and the testes were quickly removed under sterile conditions and placed in a petri dish containing Krebs-Ringer's solution with glucose. The seminiferous tubules were isolated from the interstitial tissue by careful dissection with small forceps (2). Long segments were then observed by transmitted light, using a preparative microscope with 40–100-fold magnification. A characteristic variation in the light absorption pattern permits the identification of the various stages of the seminiferous epithelial cycle (15, 25) in unstained seminiferous tubules (24). Furthermore, the accuracy of identification can be increased by taking small segments of seminiferous tubules for phase-contrast microscopic analysis of individual cells (29). Segments representing the various stages of the seminiferous epithelial cycle were then placed in small incubation chambers containing 100 μl of Krebs-Ringer's solution with glucose and 10 μCi of [^{3}H]uridine (The Radiochemical Centre, Amersham, England; sp act 29 Ci/mmol) and incubated for 2 h at 31°C under continuous shaking in an atmosphere containing 95% O_2 and 5% CO_2. The tubular segments were then rinsed several times with Krebs-Ringer's solution and either fixed for electron microscope autoradiography or chased in tissue culture for up to 36 h in the presence of nonradioactive uridine (10 μg/ml).

Tissue Culture

After labeling with [^{3}H]uridine, the material was cultured in petri dishes containing Parker-type Medium 199 with Earle's salts (Orion Oy, Helsinki, Finland) with sodium-G-penicillin (Leiras Oy, Turku, Finland) 50 IU/ml, and streptomycin sulfate (Lääke Oy, Turku, Finland) 50 μg/ml was added. No serum or growth factors were used. The tubular segments were placed on lens paper lying on a stainless steel grid at the level of the culture medium. The culture was performed most commonly for 14 h at 31°C in closed chambers in an atmosphere of 95% O_2 and 5% CO_2 (12).

Electron Microscopy and Electron Microscope Autoradiography

The tubular segments were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, for 3 h at 4°C, and then postfixed in osmium tetroxide, whereafter the samples were embedded in Epon and sectioned at 700 Å with an LKB-Huxley ultramicrotome. The sections were placed on clean glass slides coated with a thin collodion membrane (0.5% collodion in amyl acetate) and stained with 4% uranyl acetate for 30 min at 60°C and with 0.2% lead citrate at room temperature for 2–4 min. The glass slides were then coated with a monolayer of Ilford L4 emulsion by a dipping technique described by Rogers (26). The preparations were then exposed for either 1 or 4 mo at −20°C under desiccation. The autoradiograms were developed with Microdol X, fixed with Kodak Rapid Fixer, and washed several times with distilled water. The sections were then located under a stereomicroscope and carefully stripped from the collodion membrane with fine forceps and placed on copper grids. If necessary, careful poststaining with uranyl acetate and lead citrate was carried out. The observations were made with a Jeol JEM-T8 electron microscope.

RESULTS

In young spermatids (steps 1–8) the nuclei were seen to be slightly but distinctly labeled after the 2-h incubation (Fig. 1). The chromatoid body was usually unlabeled at this time, but less than 1% of the young spermatids did exhibit slight labeling (Fig. 2). When the 2-h incubation with [^{3}H]uridine was followed by a 14-h chase, the chromatoid body was distinctly labeled in all step-1–8 spermatids that synthesize RNA (Figs. 3–7). In Fig. 3, a step-7 spermatid, comparable to the cells depicted in Figs. 1 and 2, is seen after a 14-h chase. The chromatoid body is labeled and some grains are also seen in the nucleoplasm. Fig. 4 shows a step-1 spermatid after a 14-h chase. Even at this early developmental stage, the chromatoid body and the nucleus are labeled and a single grain is also seen in the space between these organelles. The situation is similar at step 5 (Fig. 5). Fig. 6 is a low-power electron microscope autoradiogram of two step-1 spermatids, in the cytoplasm of which practically no grains are seen except in the chromatoid body. The nuclei contain some grains. A high-power electron micrograph of a chromatoid body in a step-1 spermatid is seen in Fig. 7. In spite of the lobulated structure of the chromatoid body in this case, all the grains are concentrated on its dense fibrillar material. The chromatoid body of the young spermatids remained labeled when the chase was continued for up to 36 h.

Because the dense intermitochondrial material seen in the pachytene spermatocytes has been claimed to be precursor material of the chromatoid body (8, 9), the labeling of this material was also analyzed. In some cases, labeling in pachytene spermatocytes was observed over mitochondrial clusters after a 2-h incubation with [^{3}H]uridine (Figs. 8, 9). In most pachytene spermatocytes, however, the intermitochondrial material was not labeled. Furthermore, during the chase, the labeling of the intermitochondrial dense material did not increase.

DISCUSSION

It is well known from earlier autoradiographic studies that RNA synthesis stops in early sperma-

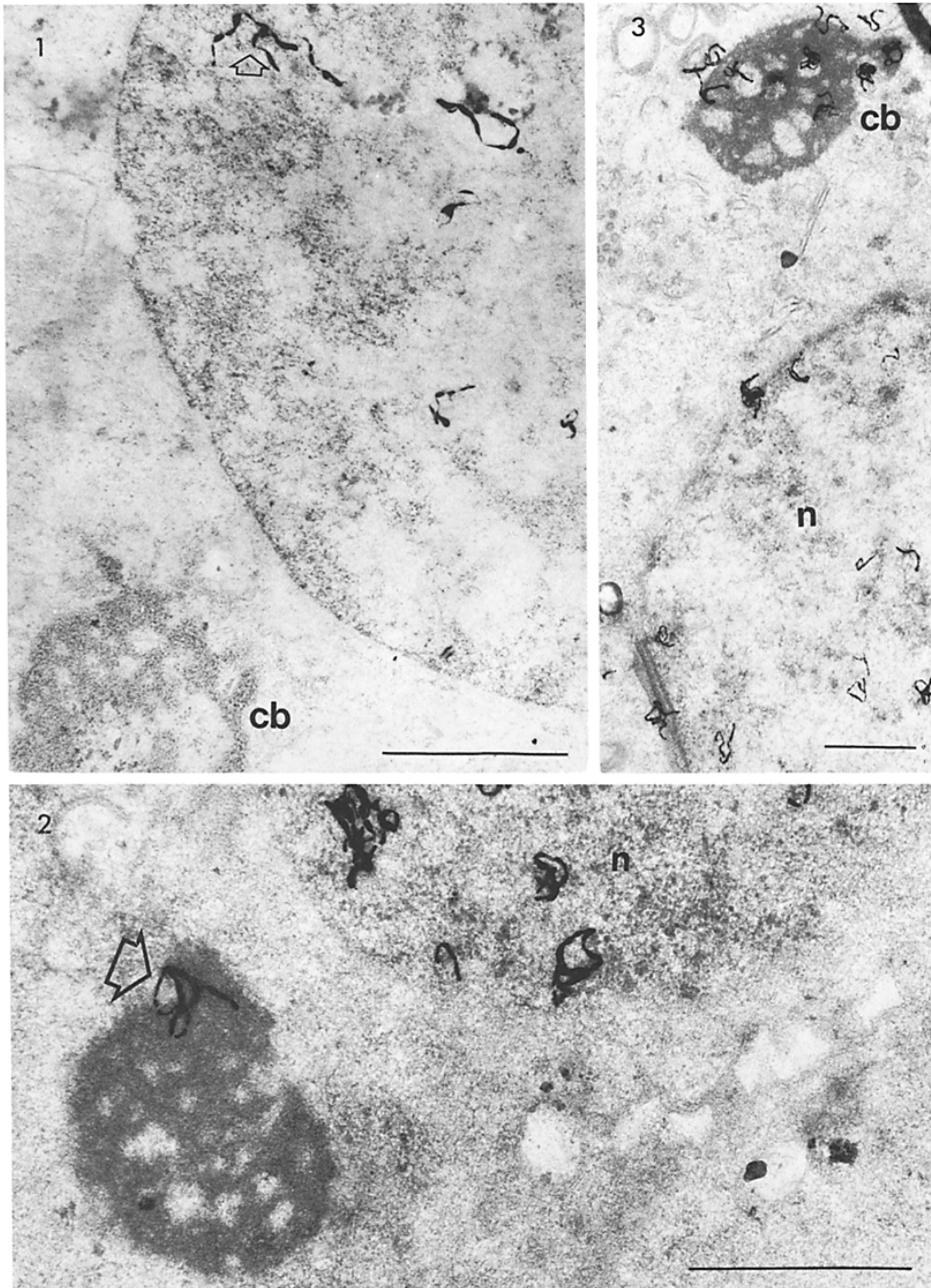

FIGURE 1 A step-7 spermatid incubated for 2 h with [^{3}H]uridine. Some grains are seen in the nucleus (arrow), but the cytoplasm and the chromatoid body *(cb)* are unlabeled. Bar: 1 μm. × 26,500.

FIGURE 2 Another step-7 spermatid after a 2-h incubation with [^{3}H]uridine. The chromatoid body is slightly labeled (arrow). The labeling of this organelle after a 2-h incubation is rare and occurs in no more than 1% of all spermatids in stages 1-8 of spermiogenesis. The nucleus *(n)* is distinctly labeled. Bar: 1 μm. × 32,600.

FIGURE 3 A third step-7 spermatid, chased for 14 h in the presence of nonradioactive uridine after 2 h of labeling. The chromatoid body *(cb)* is distinctly labeled, while some grains are seen also in the nucleus *(n)*. Bar: 1 μm. × 13,300.

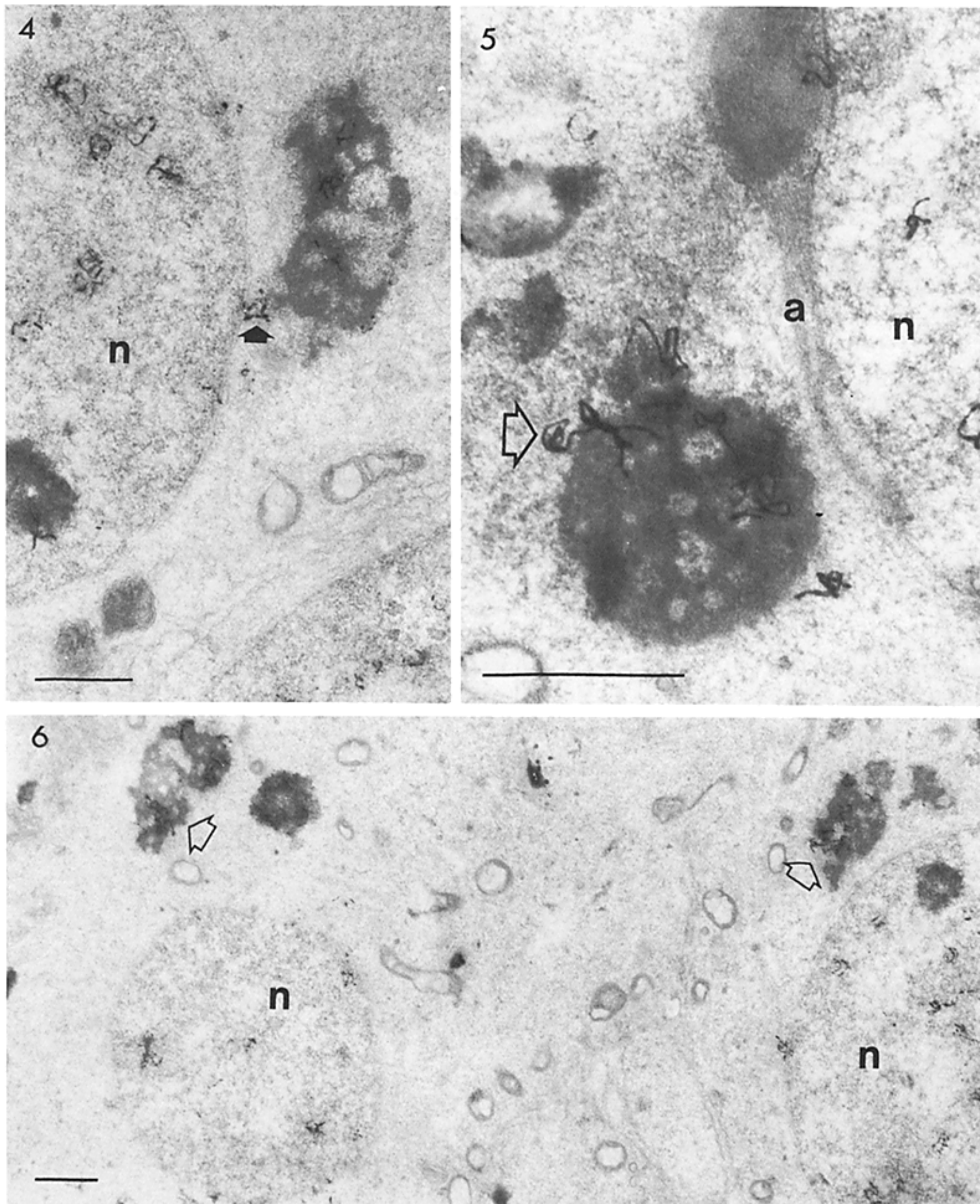

FIGURE 4 Step-1 spermatids chased for 14 h after a 2-h pulse with [^{3}H]uridine. The chromatoid body is labeled, and some grains are also seen in the nucleus *(n)*. Outside the chromatoid body no grains are seen in the cytoplasm, except in the space between the chromatoid body and the nuclear envelope (arrow). Bar: 1 μm. × 13,800.

FIGURE 5 A step-5 spermatid chased for 14 h after a 2-h pulse with [^{3}H]uridine. The chromatoid body is distinctly labeled (arrow). *a*, acrosome; *n*, nucleus. Bar: 1 μm. × 27,700.

FIGURE 6 A low-power electron microscope autoradiogram of step-1 spermatids from the same specimen as the cells in Fig. 4. Practically no grains are seen in the cytoplasm outside the chromatoid bodies which are distinctly labeled (arrows). *n*, nuclei. Bar: 1 μm. × 8,500.

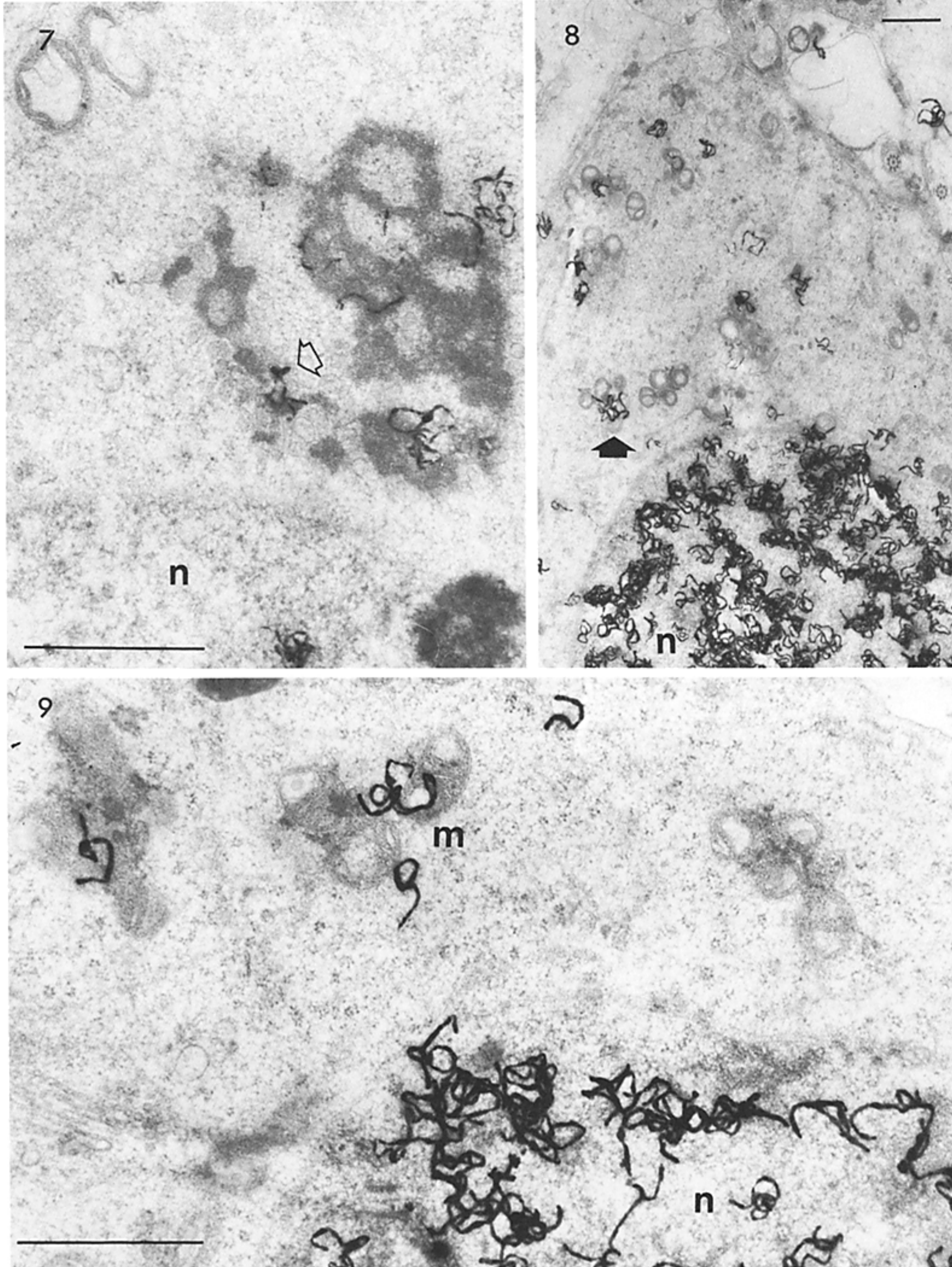

Figure 7 A labeled chromatoid body of a step-2 spermatid chased for 14 h after a 2-h pulse with [^{3}H]uridine. The grains (arrow) are mainly located over the dense fibrillar material of the chromatoid body, although in this case the body has a lobulated and irregular configuration. *n*, nucleus. Bar: 1 μm. × 25,700.

Figure 8 A pachytene primary spermatocyte from stage VII after a 2-h pulse with [^{3}H]uridine. The nucleus *(n)* is heavily labeled, but some grains are also seen in the cytoplasm. They seem to be concentrated on the mitochondrial clusters with a dense fibrillar intermitochondrial material (arrow). Bar: 1 μm. × 7,900.

Figure 9 Another pachytene primary spermatocyte treated as in the previous figure. Some grains are found over the mitochondrial clusters *(m)*, but there are also grains elsewhere in the cytoplasm. *n*, nucleus. Bar: 1 μm. × 25,700.

tids when their nuclei begin to elongate (18–21, 31). Protein synthesis, however, continues until the late steps of spermiogenesis (18, 5). Also, the most prominent morphological changes of the spermatids, the lengthening and condensation of the nucleus, growth of the tail and its associated structures, and the completion of the acrosome take place after RNA synthesis has ceased.

It is a common belief that differentiation and function of the male gamete, at least in *Drosophila melanogaster*, depend on the diploid genome and probably not on the haploid genotype of its own nucleus (16). This may be at least partly the case in mammals, too, where RNA synthesis during early spermiogenesis is considered to be too small to coordinate the remaining part of spermatid differentiation (21). In order to be able to direct protein synthesis during late spermiogenesis, the RNA must presumably have a long life-time and be stored in the cell. The labeling of the chromatoid body after [^{3}H]uridine incubation suggests that it has such an RNA storage function. The incorporation possibly begins in the pachytene stage of meiosis since the intermitochondrial dense material, which may later form the chromatoid body of the spermatids (8, 9), occasionally became labeled. Electron microscope observations also suggest that the chromatoid body is formed from material transported from the nucleus during meiotic prophase (3). Such a material transport may also occur during early spermiogenesis (30).

The character of RNA synthesis during spermiogenesis has recently been studied in the mouse by high-resolution autoradiography (14). No incorporation of the [^{3}H]uridine label was detected over the chromatoid body when the label was injected intratesticularly, even after longer survival times. This may be due to the low level of radioactivity that can be introduced into the cells in vivo. Considerably higher radioactivities can be introduced into the cells in the present in vitro experiment, and the chromatoid body was occasionally seen to be labeled after only a 2-h incubation with tritiated uridine. This may be the time required for the transport of RNA material from the nucleus to the chromatoid body.

On the basis of whole-mount electron microscope observations and the fact that the nucleolus of the spermatids was not labeled, Kierszenbaum and Tres (14) have suggested that the RNA synthesized in the spermatids is HnRNA. This is also supported by the observation that the [^{3}H]uridine labeling of the young spermatids is inhibited by α-amanitin (22). Therefore, it is likely that the label seen in the chromatoid body of the spermatids in our experiment is due to large informational RNA molecules. Attempts to characterize biochemically this RNA are currently being made in our laboratory.

The cells that are most active in synthesizing RNA during meiotic prophase are the midpachytene spermatocytes (18, 19, 13, 10, 31). We have recently shown that a major part of this RNA is composed of a special form of stable HnRNA (29), which seems to be characteristic of meiotic cells. The meiotic RNA has been shown to remain in contact with the autosomes for a long time and the main part of it is shed into the cytoplasm at the beginning of the first meiotic metaphase (19, 20), while a minor part of it is transferred to the cytoplasm during the pachytene stage. This small part may be an important constituent of the chromatoid body, as suggested by our present findings of the labeling of the intermitochondrial dense material.

Recently, it has been observed in the rat that the germ cells of both sexes have in their cytoplasm a characteristic organelle, the nuage (7). The chromatoid body is a special form of the nuage, an electron-dense organelle present in the germ cells from an early stage of their development. Cytoplasmic structures that morphologically resemble the chromatoid body have also been demonstrated in the oocytes of several insects, e.g., of the dragonfly *Cordylia aenea* L. It has been suggested that these structures are storage structures for long-lived informational RNA (11). The best studied of such organelles are the polar granules of insect oocytes that are known to contain RNA which is probably activated during embryogenesis (17). These structures could well be counterparts to the chromatoid body found in mammalian spermatocytes and spermatids as well as the cytoplasmic inclusions of golden hamster oocytes that become labeled with [^{3}H]uridine (32). All these structures may have a more general function as stores of inactive messenger RNA synthesized in the lampbrush loops of meiotic chromosomes. It seems likely that the chromatoid body is a specific cytoplasmic structure in which stable informational RNA molecules are collected and stored during the meiotic prophase and during early spermiogenesis. During late spermiogenesis when there is no RNA synthesis in the spermatids, this RNA may have a function in the subsequent differentiation of the spermatids.

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