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DIFFERENTIAL RATE OF RIBONUCLEIC ACID SYNTHESIS IN THE AUTOSOMES AND SEX CHROMOSOMES DURING MALE MEIOSIS IN THE MOUSE

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With 17 Figures in the Text

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Introduction

There is, at the present time, a concurrent evidence to indicate that genic activity is correlated with extension or unwinding of the chromosomal thread, the inactive chromosomal loci appearing as highly condensed or tightly wound up. For instance, in interphase nuclei only the dispersed chromatin appears to be active in ribonucleic acid synthesis; the condensed chromatin appears to be relatively inert. HSU (1962) and MONESI and CRIPPA (1964) have shown that in mouse somatic cells cultured in vitro incorporation of uridine-H^3 occurs only at those sites in the nucleoplasm where the chromatin has a diffuse appearance, whereas it is virtually absent in the heteropycnotic blocks or chromocenters where the chromatin is highly condensed. FRENSTER, ALLFREY and MIRSKY (1963) have shown that the condensed chromatin (heterochromatin) isolated from calf thymus lymphocytes is much less active in messenger RNA synthesis than the extended chromatin (euchromatin). The condensed chromatin contains up to 80 per cent of the total interphase DNA. This would, then, imply that most cellular genes are "repressed" as far as genetic activity is concerned. These conclusions were confirmed by electron microscopy autoradiography (LITTAU, ALLFREY, FRENSTER, and MIRSKY 1964). Another striking example of correlation between chromosome condensation and genetic inactivity concerns the X chromosome in somatic cells of the female in a variety of mammalian species. In female somatic cells, one of the two X chromosomes, which may be of either paternal or maternal origin, becomes genetically inactivated during early embryogenesis (LYON 1961, 1962; RUSSELL 1961; BEUTLER, YEH and FAIRBANKS 1962) and it appears morphologically as a prominent heteropycnotic body during interphase

(BARR and CARR 1960, OHNO and MAKINO 1961). The failure of the metaphase chromosomes to synthesize RNA both during mitosis (TAYLOR 1960; PRESCOTT and BENDER 1962; DAS 1963; FEINENDEGEN and BOND 1963; MONESI 1964a, b; MONESI and CRIPPA 1964) and during meiosis (TAYLOR 1958; HENDERSON 1964; MONESI 1964a, b; MUCKENTHALER 1964) was also interpreted to result from the inability of the contracted chromosomal thread to act as a "template" for RNA synthesis.

Possibly, the most remarkable evidence of association between genic activity and unwinding of the chromosomal thread concerns the studies on RNA metabolism in the "lampbrush" chromosomes of amphibian oocytes (GALL and CALLAN 1962; IZAWA, ALLFREY and MIRSKY 1963) and in the giant chromosomes of many dipteran larvae (see, as a review reference, BEERMANN 1963). These studies have shown that RNA synthesis occurs only or mainly in certain regions of the chromosomes where the DNA is uncoiled to form special structures named, respectively, "loops" and "Balbiani rings" ("puffs"), whereas it is absent or slow along the chromosomal axis where the DNA is tightly coiled and folded into chromomeres.

Since during most of male meiosis in the mouse, as well as in most animal species, the sex chromosomes show positive heteropycnosis relative to the autosomes, it appeared of interest to investigate comparatively the pattern of ribonucleic acid synthesis in the autosomes and in the sex chromosomes using tritium-autoradiography and squash preparations of isolated tubules. HENDERSON (1964) has recently reported that the X univalent is inactive in RNA synthesis during male meiosis in the grasshopper and locust.

Material and Methods

C3H male mice, 12 to 14 weeks old, were used. Uridine-H^3 (The Radiochemical Centre, Amersham; specific activity 2.1 C/mM) was used as a precursor of RNA. Since it was previously observed that, when uridine-H^3 or cytidine-H^3 are injected intraperitoneally or intravenously, the labeling is confined to the peripheral tubules of the testis (MONESI 1964a, b), the precursor was now injected directly into the testis under the albuginea at a dose of 6 μc per testis. Preliminary autoradiographic experiments had shown that under these conditions the precursor diffuses very rapidly throughout the whole organ resulting in equal labeling of the peripheral and the central tubules. At various intervals after injection, the animals were killed, the testes were freed from the albuginea, placed in a Petri dish containing 0.7 per cent hypotonic citrate solution, and the testicular tubules were teased apart. After 15—20 min of hypotonic treatment, the isolated tubules were either (a) fixed for 10 min in alcohol—acetic acid (3:1), stained for 20 min with lactic-acetic orcein and squashed on slides, according to the procedure of WELSHONS, GIBSON and SCANDLYN (1962), then extracted with 5 per cent trichloroacetic acid (TCA) at 0°C to remove unincorporated precursor, washed two hours in running water and autoradiographed, or (b) they were fixed as described above, then squashed

on slides with the aid of few drops of 50 per cent lactic acid, extracted with cold TCA, autoradiographed and stained with hematoxylin through the autoradiographic emulsion after processing. A few samples were fixed in Orth's fixative, embedded in paraffin, sectioned at 2 μ, and stained with periodic acid-Schiff and hematoxylin. The autoradiographs were prepared by using Kodak NTB2 liquid emulsion and were exposed in light-tight boxes at 4^{0}C from 20 to 100 days. In order to test the specificity of isotope incorporation, some squashes were incubated with 0.1 per cent ribonuclease (Light, 5 $\times$ crystallized) in distilled water at 40^{0}C for 4 hours, then washed in running water and autoradiographed. The enzymatic treatment removed practically all the label incorporated into meiotic cells and Sertoli cells.

Observations

The autosomes

The autosomes actively synthesize RNA during great part of meiotic prophase. The rate of uridine-H^3 incorporation, as judged by uridine-H^3 labeling 30 to 60 min after injection, varied, however, considerably during the course of the meiotic cycle. It was rather low during the preleptotene resting stage — when DNA is synthesized (Monesi 1962) — then ceased or fell to a very low level during early prophase until early pachytene. After these stages, the rate of incorporation increased rapidly to a maximum in middle pachytene, but declined again progressively during late pachytene, diplotene and diakinesis, as chromosome coiling progresses, and stopped completely during late diakinesis, first metaphase and anaphase. Secondary spermatocytes were very little labeled during the short resting stage and completely unlabeled during metaphase and anaphase (Figs. 1—14).

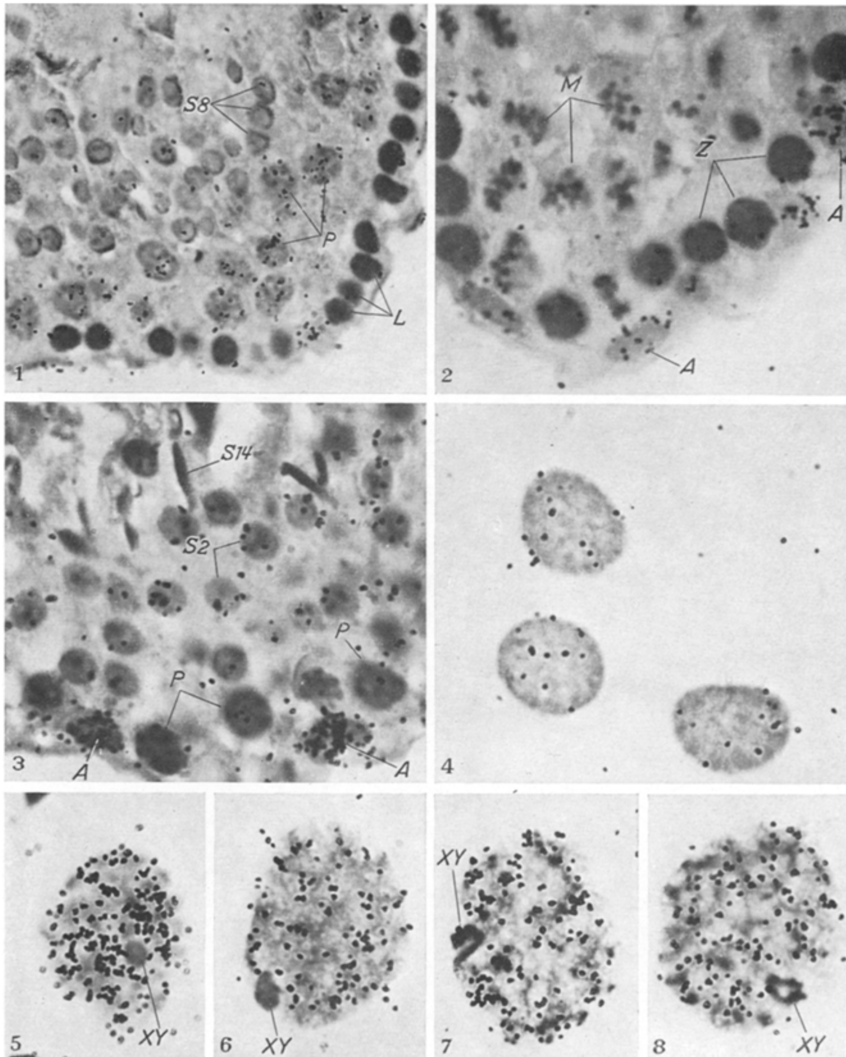
In pachytene nuclei, the silver grains were seen to overlie exclusively the chromosomal threads up to 2—3 hours after injection (Figs. 10—12). It was about 3 to 4 hours after injection before a detectable amount of radioactivity appeared in the cytoplasm. With increasing times after labeling, the more and more of nuclear synthesized RNA appeared in the nuclear sap and in the cytoplasm. However, the ribonucleic acid associated with the chromosomes at diplotene is not retained by them through division, but is suddenly released to the cytoplasm as the cell enters diakinesis and metaphase. This is inferred by the observation that (a) whereas at pachytene stage the labeling was exclusively chromosomal up to 2—3 hours after injection, at diplotene and diakinesis a fair amount of label was already in the cytoplasm 30 min after injection (Fig. 13), (b) at longer post-injection intervals (4 to 12 hours), as labeled pachytene and diplotene nuclei develop to metaphase stage, this latter stage also appeared labeled but the silver grains were present almost exclusively over the cytoplasm, whereas in pachytene nuclei the labeling was still overwhelmingly chromosomal. As a result of this rapid discharge of chromosomal RNA to the cytoplasm during late meiosis, metaphase

chromosomes appear to contain little or no RNA. Analogous evidences of a rapid dispersion of chromosomal RNA into the cytoplasm at metaphase were reported by a number of authors in mitotic cells (TAYLOR 1960; PRESCOTT and BENDER 1962 and 1963; FEINENDEGEN and BOND 1963; MONESI 1964a, b; MONESI and CRIPPA 1964). Since this rapid release of nuclear RNA to the cytoplasm at very late meiotic prophase takes place after the nuclear membrane has disappeared, it seems reasonable to conclude that it may involve a dissociation from the chromosomes rather than merely depending upon the removal of the nuclear membrane as a barrier.

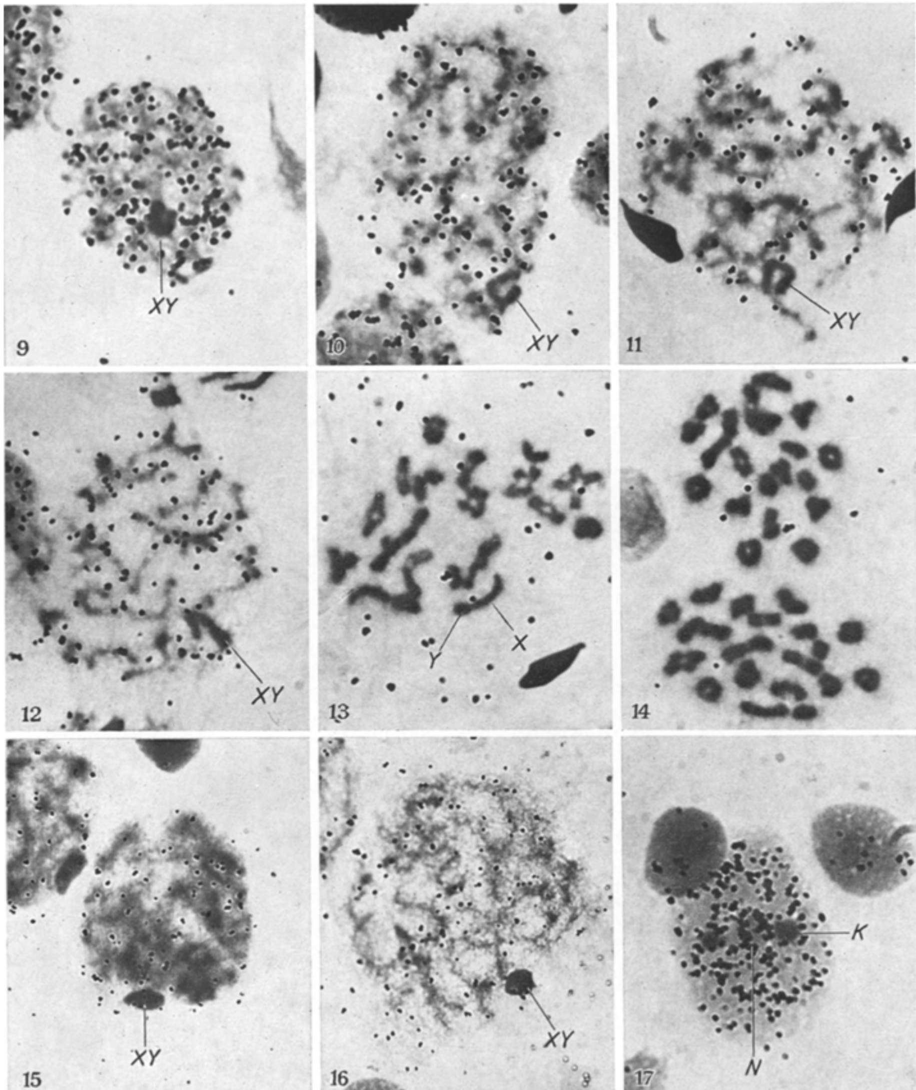
The finding of absence or great depression of RNA synthesis during early meiotic prophase, when the chromosomal threads are largely uncoiled, followed by intense synthesis during pachytene, concomitantly with a progressive increase in chromosome condensation, was unexpected since in mitotic chromosomes the rate of RNA synthesis is high during early prophase and then gradually declines as the process of chromosome coiling progresses (TAYLOR 1960; PRESCOTT and BENDER 1962; FEINENDEGEN and BOND 1963; MONESI 1964a, b; MONESI and CRIPPA 1964). Leptotene and zygotene nuclei were even less labeled than the contracted bivalents at late pachytene and diplotene (Figs. 4, 11, 12). In a previous research carried out on tissue sections (MONESI 1964a, b), it was observed that meiotic RNA synthesis ceases or reaches an insignificant level soon after the completion of premeiotic DNA synthesis for a period of about 140 hours, that is for about one half of the total length of meiosis, as judged from the duration of the seminiferous cycle (OAKBERG 1956). A similar drop of RNA synthesis during early meiotic prophase was not reported to occur in the grasshopper and locust (HENDERSON 1964, MUCKENTHALER 1964) and in plants (TAYLOR 1959, HOTTA and STERN 1963). However, in *Tulbaghia violaceae* microsporocytes a cessation or a drop of RNA synthesis was reported to occur at the time of DNA replication and at the zygotene pairing stage (TAYLOR 1958).

The sex chromosomes

During most of male meiosis in the mouse, as well as in other species, the sex chromosomes are allocyclic relative to the autosomes. They are tightly coiled up and positively heteropycnotic throughout leptotene, zygotene and most of pachytene stages. During these stages they are enclosed within the so called sex vesicle which appears as a positively heteropycnotic body applied to the nuclear membrane. At early diplotene the vesicle gradually disintegrates, and the sex chromosomes clearly appear as two separate bodies connected end-to-end (MATTHEY 1953, OHNO, KAPLAN and KINOSITA 1959, GEYER-DUSZYŃSKA 1963). At



Figs. 1—3. 3 μ sections of seminiferous tubules labeled for 1 hour with uridine-H^3 and stained with periodic-acid Schiff and hematoxylin. Focus on the grains. Abbreviations: *A* type A spermatogonia. *L* leptotene. *M* meiotic metaphase. *P* pachytene. *S2*, *S8*, *S14*, spermatids at various steps of spermiogenesis. *Z* zygotene. — The various generations of germ cells are arranged from the basement membrane (at the bottom of the figures) to the lumen of the tubule (at the top of the figures) according to their age. — Fig. 1, stage VIII, ca. 800. Fig. 2 stage XII, ca. 1200. Fig. 3, stage II, ca. 1200. — Note (a) the absence of RNA labeling in the generation of young spermatocytes at leptotene (Fig. 1), zygotene (Fig. 2), and early pachytene (Fig. 3) stages at the periphery of the seminiferous tubule, (b) the presence of a significant RNA labeling in the older generation of primary spermatocytes at middle pachytene in the center of the tubule in Fig. 1, and (c) the absence of label over meiotic metaphase figures in Fig. 2. Figs. 4—8 are squashes of primary spermatocyte nuclei labeled for 1 hour with uridine-H^3. Lacticacetic orcein or hematoxylin. ca. 1500. X, the X chromosome; Y, the Y chromosome; XY, the X and Y chromosomes. — Fig. 4, zygotene or very early pachytene, very little labeled. Figs. 5—8, pachytene, heavily labeled over the autosomes and completely unlabeled over the heteropycnotic sex chromosomes



Figs. 9—14. The distribution of RNA labeling at various stages of male meiosis 30 to 60 min after injection of uridine-H^3. Lactic-acetic orcein or hematoxylin staining. ca. 1500. For abbreviations see Figs. 4—8. — Figs. 9 and 10, pachytene. Figs. 11 and 12, late pachytene. Fig. 13, diakinesis. Fig. 14, first metaphase figures. — Note (a) the absence of label over the sex chromosomes at all stages of meiosis and over the autosomes at metaphase, and (b) the presence of abundant cytoplasmic labeling at diakinesis.

Figs. 15 and 16, are pachytene nuclei labeled for 1 hour with arginine-H^3. Hematoxylin. ca. 1500.— Note the presence of label over both the autosomes and the sex chromosomes

Fig. 17. Nucleus of a Sertoli cell labeled for 1 hour with uridine-H^3. Hematoxylin. ca. 1500. Note the high density of grains over the nucleolus (*N*) and little or no labeling over the heteropycnotic chromocenters or karyosomes (*K*) flanking the nucleolus

diakinesis and metaphase I and II the sex bivalent becomes isopycnotic to the autosomes. Although clearly visible only beginning with the diplotene stage, the end-to-end association of the X and Y chromosomes is already well established during pachytene, as revealed by ribonuclease digestion (OHNO, KAPLAN and KINOSITA 1959).

In contrast to the autosomes which were labeled throughout the second half of meiotic prophase, the heteropycnotic sex chromosomes were invariably unlabeled at all stages of the meiotic cycle. They remained unlabeled also during diplotene and diakinesis, when they become isopycnotic to the autosomes, whereas the autosomes at these stages still exibited a certain amount of labeling (Figs. 5—14). It is noteworthy that, unlike RNA precursors, amino acids-H^3 were incorporated also in the sex chromosomes at all stages of meiosis (Figs. 15, 16).

Discussion

The failure of the heteropycnotic sex chromosomes to incorporate RNA precursors at all stages of meiosis is interpreted to indicate that the sex chromosomes are inactive in messenger RNA synthesis during male meiosis. This finding is, then, in line with the current view that only dispersed chromatin is active in the production of messenger RNA. In this connection, it is of interest to note that the supporting Sertoli cells were always found to be heavily labeled over the nucleolus and the nucleoplasm except over the two highly condensed chromocenters on each side of the nucleolus (Fig. 17).

It seems, however, that the degree of chromosome contraction *as such* is not the only condition which governs the rate of RNA synthesis during meiosis, since in the mouse the autosomes were found to be either inactive or very little active in RNA synthesis during early prophase (leptotene to early pachytene), when the chromosomes are largely uncoiled, and progressively more active during middle pachytene in spite of a great increase in chromosome coiling. During late pachytene to diakinesis, as the chromosome coiling becomes the more and more complete, the rate of synthesis decreases rapidly to eventually stop at metaphase, but even the contracted bivalents at late pachytene and diplotene were more labeled than the dispersed chromatin at leptotene and zygotene stages.

A possible explanation of this unusual behavior may be suggested from the experiments on RNA metabolism in the "lampbrush" chromosomes of amphibian oocytes at diplotene stage. Incorporation studies have shown that in the chromosomes of newt oocytes RNA synthesis is very intense in the lateral loops and is absent or slow in the dense chromosomal axis (GALL and CALLAN 1962, IZAWA, ALLFREY and

MIRSKY 1963). Kinetics studies of deoxyribonuclease-induced breaks (GALL 1963) and enzymatic digestion experiments (MACGREGOR and CALLAN 1962) have led to the concept that the lampbrush chromosome is equivalent to two closely apposed DNA double helices tightly coiled and folded into chromomeres along the axis and stretched out into paired loops laterally. These studies have led to the view that the "looping out" of the DNA molecule from the dense chromosomal axis is the mechanism allowing RNA synthesis to occur in a given chromosomal locus. Although the presence of DNA lateral projections from the main chromosomal axis has been clearly demonstrated only in amphibian oocytes, in virtue of their great size, it is conveivable that this lampbrush organization of the chromosomes is a general feature in mitotic and meiotic chromosomes whenever a chromosomal locus undergoes RNA synthesis. In this sense, the "looping out" of primer DNA would be a general prerequisite to allow RNA synthesis. Lateral projections similar to the loops of amphibian oocytes chromosomes have been, for instance, repeatedly described in grasshopper spermatocytes (RIS 1945, HENDERSON 1964). In mammalian chromosomes, lateral projections are more difficult to be observed. However, late prophase chromosomes in mammalian male meiosis frequently exibit a fuzzy outline (Fig. 16; see also Figs. 2—5 in OHNO, KAPLAN and KINOSITA 1959). Furthermore, electron microscopic observations (MOSES 1960, 1963; SOTELO and TRUJILLO-CENÓZ 1960) have shown that the bivalent chromosomes of rat and insect primary spermatocytes exibit numerous kinked microfibrils radiating from the chromosome axis, that might well be loop structures, as suggested by MOSES (1964). The frequent occurrence of RNA labeling over the chromosomal margins during male meiotic prophase in the mouse (Figs. 10—12) may also be interpreted to result from the presence of lateral projection of DNA loops.

The degree of lampbrush organization of the chromosomes seems to be, to a certain extent, independent of the general process of chromosome contraction *per se*. In fact, maximal lampbrush organization of the chromosomes in newt oocytes (CALLAN and LLOYD 1960) and in orthopteran spermatocytes (HENDERSON 1964) is attained at diplotene stage, when the chromosomes are highly condensed. Then, *a measure of chromosome activity would be the extent of loop organization rather than the degree of coiling of the main chromosomal axis.* In fact, the biochemical studies of DAVIDSON, ALLFREY and MIRSKY (1964) have demonstrated that most of the RNA produced during oogenesis in *Xenopus laevis* is synthesized during the diplotene lampbrush stage.

On the basis of these evidences, it may be suggested, as a working hypothesis, that in the mouse primary spermatocytes DNA loops are absent from early prophase chromosomes and that a lampbrush organi-

zation develops late in prophase. Leptotene and zygotene chromosomes, in spite of the low level of chromosome coiling, would thus be incapable of RNA synthesis; synthetic activity would commence and progressively increase later during the pachytene stage, concomitantly with the appearance and development of loop structures. This hypothesis would also account for the persistance of a certain level of RNA synthesis in the contracted bivalents at diplotene and diakinesis (our observations and HOTTA and STERN 1963, HENDERSON 1964), as well as in early metaphase chromosomes of mouse spermatogonia (MONESI 1964a, b) and mammalian somatic cells cultured in vitro (KONRAD 1963, MONESI and CRIPPA 1964).

Summary

1. Ribonucleic acid synthesis was studied in the autosomes and sex chromosomes during male meiosis in the mouse using uridine-H^3 labeling and autoradiography.

2. In the autosomes, the incorporation of uridine-H^3 into RNA ceased or fell to an insignificant level during early meiotic prophase until early pachytene. After these stages, the rate of incorporation increased rapidly to a maximum in middle pachytene, but declined again progressively during late pachytene, diplotene and diakinesis, as chromosome coiling progresses, and stopped completely during metaphase and anaphase. Secondary spermatocytes were very little labeled during the short resting stage and completely unlabeled during metaphase and anaphase.

3. In contrast to the autosomes, the heteropycnotic XY bivalent was invariably unlabeled throughout the meiotic cycle.

4. A working hypothesis is forwarded to account for the absence or scarcity of RNA synthesis during early meiotic prophase.

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