ASYNCHRONOUS REPLICATION OF DNA IN A HETEROCHROMATIC SET OF CHROMOSOMES IN *PSEUDOCOCCUS OBSCURUS*^{1,2}

DEE BAER

Biology Department, San Diego State College, San Diego, California

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THE replication of chromosomal DNA has been shown to be asynchronous for whole chromosomes or for parts of a single chromosome by LIMA-DE-FARIA (1959, 1961), MORISHIMA, GRUMBACH, and TAYLOR (1962), TAYLOR (1960), WIMBER (1961), WIMBER and PRENSKY (1963), and others. The chromosomes of the organisms studied generally contained heterochromatin. This heterochromatin is genetically determined in the sense that certain parts of the genome, such as sex chromosomes, display heteropyknosis during all or some phases of the life cycle.

In a majority of the cases, heterochromatin has shown asynchrony of DNA synthesis with respect to the rest of the genome, although some parts of the euchromatin have also been found to replicate asynchronously.

This paper reports autoradiographic studies with tritiated thymidine on mealy bugs of the species *Pseudococcus obscurus* Essig. The results show that the magnitude of genetic material involved in asynchronous replication may extend to an entire complement of heterochromatic chromosomes, for which a prior history as euchromatin is known.

In mealy bugs, both sexes are diploid and there are no distinguishable sex chromosomes. The sexes are, however, dramatically different: during embryogenesis in the male the paternal set of chromosomes becomes heterochromatic and remains so throughout his life. The maternal chromosomes remain euchromatic. The paternal chromosomes degenerate after spermatogenesis; the sperm contain and transmit only the maternal chromosomes. Thus the euchromatic set in the male of one generation becomes the heterochromatic set in the males of the next. This situation was recently reviewed by BROWN and NUR (1964).

Concurrent with this change to heterochromatin is a marked change in genetic function. The chromosomes become largely genetically inert as measured by radiation response (BROWN and NELSON-REES, 1961) and other means.

The present study was undertaken to determine if this newly acquired, or facultative, heterochromatic state shows asynchronous replication similar to that of the genetically determined heterochromatin of other organisms. The results indicate that an asynchrony of the maternal, euchromatic (E) and paternal, heterochromatic (H) sets does indeed exist and that the E set precedes the H set

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in replication. The transmission of chromosomes from father to son involves a shift to heterochromatin in association with a shift to late DNA synthesis.

MATERIALS AND METHODS

Test organism: Mealy bugs of the species *Pseudococcus obscurus*, with $2N=10$, were studied. These Homopteran insects feed on plant juices and are routinely cultivated, at room temperature, on sprouting potatoes in ventilated jars.

Testicular and abdominal diploid tissues of the second nymphal stage were investigated.

Administration of H^3TDR : Since mealy bugs suck plant juices, it was possible to administer the tritiated thymidine (H^3TDR) in the following manner: (1) clean potatoes with apical sprouts less than $\frac{1}{8}$ inch high—but otherwise smoothly surfaced—were selected. All but about five apical sprouts were scraped off and these ex-sprout areas covered with liquefied paraffin. (2) Fifty young *P. obscurus* were then added to the potato in the culture jar and allowed to settle on the sprouts. (3) Four tunnels at 90 degrees to each other were made with a dissecting needle under each remaining sprout to a depth of $\frac{1}{4}$ to $\frac{1}{2}$ inch, at an angle to the surface of about 45 degrees. (4) H^3TDR , (New England Nuclear Corp., 575 Albany St., Boston 18, Mass.) as thymidine-methyl- H^3 , at a concentration of 1 mc/ml (maximum specific activity used was 5.15 c/mm) was injected into all tunnels. Each prepared potato received 40 to 60 λ of H^3TDR .

This method allowed the mealy bugs to take in the H^3TDR while feeding on the sprouts because the net movement of the injected H^3TDR is upward through the phloem into the growing sprouts. The presence of labeled nuclei in autoradiographs prepared from the young potato leaves showed that not all of the H^3TDR reached the insects.

Collection times were 1.5, 3, 4.5 and 9 hours following administration of H^3TDR .

Direct injection of H^3TDR into the second nymphal stage animals was not feasible since they are small (less than 1 mm long), soft bodied, and covered with a waxy meal.

Autoradiography: The general procedure was fixation in 4 parts chloroform : 3 parts absolute ethanol : 1 part glacial acetic acid (v/v/v) at various times after administration of H^3TDR . The insects were then changed to 3:1 (absolute ethanol : glacial acetic acid) for at least 6 hours, followed by distilled water for one day.

The tissues of interest were dissected out onto albuminized slides in 45% acetic acid, warmed briefly, and then the preparations were squashed, care being taken that the cells were well distributed and evenly flattened. The slide preparations were frozen on dry ice and the coverslips flicked off. After this the slides were set in absolute ethanol for 15 min, dried in air, then changed through 95%, 70%, and 30% ethanol to water before filming.

In some experiments, nucleases were used on comparable pieces of tissue from a given individual. Three slides were made per individual and carried through the procedures given above. The first slide received no enzyme treatment, but instead was incubated 3 hours in 0.1% gelatin (w/v) in 0.003 M $MgSO_4$ at pH 6.5 and then returned to distilled water before filming. The second was treated for 3 hours with a 0.1% solution of DNase (Sigma Chemical Corp., 3500 DeKalb St., St. Louis, Mo.) in 0.003 M $MgSO_4$ with 0.1% gelatin, adjusted to pH 6.5. The third slide was treated with a 0.02% solution of RNase (Sigma) at pH 5.5 for 3 hours. Following nuclease treatment, slides were immersed 5 min in 5% trichloroacetic acid at 4°C. All treatments were given at room temperature except as noted.

RNase did not remove the H^3TDR label. When DNase was used on H^3TDR treated tissues, no labeling of autographs was observed except in one series. In that series, following DNase treatment, the label was not removed. However, it was concluded that the DNase preparation used in that series was inactive because the cytological appearance of the nuclei was no more diffuse than in the control slides.

Autoradiographic stripping film (AR-10) from Kodak, Ltd., England, was applied in the usual way. Exposure time was 2 to 3 weeks. Preparations were developed and fixed in accordance with standard autoradiographic procedures, allowing 12 min each for developing and fixing.

Stains: Feulgen staining was unsuccessful for unknown reasons. The most successful stain

tried was a 2% aqueous solution of pyronin Y (E. GURR, London, England) extracted three times with equal volumes of chloroform. After 15 min in the stain, the autoradiographic slide preparations were washed and air dried.

RESULTS

Differential nuclear labeling: Tissues were fixed at short intervals after administration of H^3 TDR to the potato on which the mealy bugs fed. In autoradiographs prepared from somatic tissues of second nymphal stage males fixed after feeding for 1.5 hours, only a few large interphase nuclei were lightly labeled. Such large, endopolyploid nuclei typically contain no heterochromatin; they were never observed to be dividing or to be heavily labeled. In males fixed at 3 hours, interphase nuclei often showed label clustered exclusively over the E (euchromatic) or over the H (heterochromatic) complement; prometaphase nuclei with chromosomes individually distinguishable also often showed label only over the H or E sets; metaphase nuclei, particularly in animals fixed after 3 to 4.5 hours feeding, showed label restricted to one side or to the middle of the plate of congressed chromosomes (Figure 1). This last phenomenon reflects the nonrandom lateral distribution of the E and H sets typically observed on the metaphase plate.

Among labeled interphases from animals fixed at 3 hours, 54% of the somatic, H-containing nuclei showed only the H set labeled (Figure 1) whereas about 39% showed only the E complement labeled (Figure 2). The remainder, 7%, showed label over both E and H sets (Figure 3).

These data suggest DNA asynchrony between the two complements. Asynchrony can be definitely established on the basis of these data if in the animals fixed at 3 hours, H^3 TDR was available for only a short part of the cell generation time.

Since few labeled nuclei were observed in animals fixed at 1.5 hours, H^3 TDR was available for a maximum of 1.5 hours in those animals fixed after feeding for 3 hours. As will be discussed later, it appears likely that 1.5 hours is only a small part of the cell generation time.

On the basis of the data from animals fixed after 3 hours it can therefore be concluded that the H and E sets synthesize DNA asynchronously with H-DNA synthesis occupying at least 54% of the S period (the time interval of DNA synthesis) and E-DNA synthesis at least 39%.

Among somatic interphases from individuals fixed at later times, up to 9 hours, the percentage of nuclei labeled exclusively in E changed very little, but the percentage labeled in H decreased while the percentage labeled in both sets increased. The time sequenced data are presented in Figure 5. As the frequency of interphases labeled in both sets increased, the frequency of labeled metaphases increased (compare Figures 5 and 6).

The assumption has been made that the mitotic, somatic population of cells considered thus far is randomized with respect to cell division. No temporal order of DNA synthesis for E and H can be elucidated by observations of interphase labeling patterns in such a population. Yet this question must now be confronted.

FIGURES 1-3.—Second-instar male somatic cells. FIGURE 1.—Metaphase (M) plate, labeled to one side. Interphase with label only over heterochromatin (H). Fixed at 4.5 hours. $\times 2100$. FIGURE 2.—Interphases. Label only over euchromatin (E) at bottom; Only over H at top. Fixed at 3 hours. $\times 1400$. FIGURE 3.—Label over both H and E in cell at top. Fixed at 4.5 hours. $\times 1750$.

FIGURE 4.—Second-instar male spermatogonial mitoses. Early prophase (EP) labeled in H only, euchromatin not visible. Late prophase (LP) not labeled, five H chromosomes condensed, E not visible. Prometaphase (PM) not labeled, H in center and five E chromosomes peripheral. Fixed at 4.5 hours. $\times 2000$.

FIGURE 5.—Distribution of interphase label between the E and H complements. Collection times were at 1.5, 3, 4.5 and 9 hours after administration of H^3TDR . At least 100 nuclei were counted for each collection point. Cells fall into three classes relative to labeling pattern: the class with label in H only, the class with label in E only, and the one with label in both E and H.

FIGURE 6.—Distribution of labeled and unlabeled somatic division figures (males plus females) at various times after administration of H^3TDR given in terms of mean number of cells in a given stage per animal scored. Open symbols, unlabeled division figures; black symbols, labeled division figures. ○, metaphases; △, anaphases; □, telophases. At least ten animals were scored for each collection point.

The question is whether the E set precedes or follows the H set in chromosome replication.

Since the testis is a parasynchronous tissue it was used to study this question. Early and late prophases and a prometaphase from the testis of a male fixed at 3 hours are shown in Figure 4. Only the early prophases are labeled; and they are labeled only in the H set. Later prophases and one prometaphase without label can be seen. Note that no E sets are labeled at all. No other stages of the spermatogonial mitotic cycle occurred in this testis.

If the E set is replicated before the H set, the time between H replication and the next prophase will obviously be shorter than that between E replication and prophase. The above observation, in which only the H set is labeled at prophase shortly after exposure to tritiated thymidine thus conforms to the idea that replication of the E precedes that of the H set. The observation is incompatible with

the idea that E follows H in replication since in that case E labeling is later prophases in addition to the H labeling of early prophases should have been seen. This was not seen.

Spermatogonial interphases with label in E only, in H only, and in both sets, were found together in testes of other males fixed a few hours after administration of H^3TDR . However, none of these testes were analyzable as to the order of E and H in DNA synthesis.

Autoradiographs of tissues from second nymphal stage females were made and examined under exactly the same conditions as obtained for males. These preparations of female cells often showed labeled interphases and mitotic stages. The interphases never showed clustering of label in any part of the nuclei, i.e., the label always appeared uniformly distributed. Prometaphases with labeled, countable chromosomes showed all 10 chromosomes uniformly labeled. In metaphases the label was also uniformly distributed across the plate.

Mitotic cycle: It is important to ascertain if the mitotic cycle in mealy bugs is sufficiently long so that the observations of differential labeling between H and E which led to the notion of H-E asynchrony can be regarded as valid.

Mitotic cycles have been examined in many organisms and tissues by autoradiographic methods by CRONKITE, BOND, FLIEDNER, and RUBINI (1959), HOWARD and DEWEY (1960), PELC and LACOUR (1960), and others. However, the condition of growth of mealy bugs are not conducive to such a study. Some of the difficulties encountered will be discussed later. In addition, labeled nuclei were scored from various abdominal tissues; the assumption was made that these tissues were comparable in mitotic activity. Mitotic cycle estimations presented here are only indicative.

The interval from the end of S up to metaphase is easily estimated in this material. As shown in Figure 6, it is 1.5 hours (3.0 to 1.5). If cell division (D) is taken to consist of metaphase, anaphase, and telophase, the minimum D is then the interval from first labeled metaphase to first labeled telophase. Based on Figure 6, this would be 1.5 hours (4.5 - 3.0).

In the division figures seen metaphases comprised 63% of the total (Table 1). Assuming that the frequency of metaphases among all D's is proportional to the duration of metaphase, a second estimate of D can be obtained. It is 1.5 hours

TABLE 1
Frequencies of stages among the observed division figures

Time	N	D	Metaphase	Anaphase	Telophase
1.5 hr	4	27	.67	.22	.11
3.0 hr	5	46	.57	.30	.13
4.5 hr	12	81	.62	.26	.12
9.0 hr	14	53	.70	.24	.06
Average:			.63	.26	.11

Time, interval between administration of H^3TDR and fixation; *N*, number of male and female individuals in which division figures were observed; *D*, total number of division figures observed as defined in text.

from first labeled metaphase to first labeled anaphase in Figure 6. If metaphase is $0.63D$ and lasts 1.5 hours, then D is $1.5/0.63$ or about 2.4 hours.

D has been found by others to be in the range of 4 to 13% of the average cell generation time, G , for rapidly dividing tissues (see for instance CRONKITE, BOND, FLIEDNER, and RUBINI 1959; HOWARD and DEWEY 1960; WIMBER 1959). Lower and upper limits on G can therefore be estimated for the present system, and an indication of the duration of S derived from this estimate. If D is taken as 2.4 hours, the lower limit of G is $2.4/0.13$ or about 18 hours. The upper limit is about 60 hours.

S can be estimated as a fraction of G by the frequency of labeled, mitotically active interphases per animal after a short exposure to H^3TDR . Four males fixed at 4.5 hours were used. Somatic cells containing an H set were scored; it was assumed they are all mitotically active. Females were not used because all nuclei are completely euchromatic.

Approximately 200 interphases were scored per individual. The range of labeled interphases for these four individuals was 11% to 24%. The mean was 17%. This indicates, tenuously, the percent of cells in S , and thus S can be estimated as 17% of G . Therefore the minimum estimate of S is 3 hours, the maximum is 10.

In conclusion, the aspects of the mitotic cycle on which some information is available in abdominal, somatic tissues of mealy bugs as a result of these studies are (listed in decreasing order of certainty): (1) the interval from S to D is a minimum of 1.5 hours, (2) D is 2.4 hours, or between 1.5 and 2.4 hours, (3) G is in the range of 18 to 60 hours, and (4) S takes 3 to 10 hours.

These estimates on the mitotic cycle are consistent with the notion that the differential labeling of H and E observed in 3 hours fixations is due to H - E asynchrony of DNA synthesis.

The search for chromosome breakage: In some organisms administration of H^3TDR has been observed to increase chromosome breakage (e.g., WIMBER 1959).

In the present studies, labeled cells were carefully examined for chromosome breakage. Since the chromosomes of mealy bugs are holokinetic in regard to spindle activity, even small fragments can move normally in anaphase (BROWN and NUR 1964). Therefore, if any fragmentation had occurred it would have been readily observed in appropriate cells. None was detected. Furthermore, no other types of aberrant mitotic figures were observed.

DISCUSSION

Late, asynchronous H replication in terms of mitotic events: In the parasynchronous testicular tissue of one animal, only prophases through prometaphases were present. Among these, label was found only in the earliest prophases and then only in the H sets. Hence it was possible to say that the asynchrony of DNA synthesis between E and H is such that E precedes H .

The asynchrony established in somatic tissues can be accounted for by the

same sequence of replication. The large drop in the frequency of somatic interphases labeled in H only, coupled with the rise in the frequency of interphases labeled in both sets, with time (Figure 5), is understandable only if E precedes H in synthesis. Hence with longer exposure to H^3TDR (3 to 4.5 hours fixations), more cells make the transition from the E to the H compartment of S while the isotope is available. Consequently, more nuclei show a label in both sets. This would be detrimental to the E-labeled class except that simultaneously more cells are entering the E compartment of S from the pre-S interphase period. This means that the E-labeled class is expected to stay constant, and, as seen in Figure 5, very little drop in this class was observed.

The class of cells labeled in H only decreases during the exposure period from 3 to 4.5 hours because more cells leave the H compartment of S and enter mitosis. Thus a rise in labeled metaphases is observed at this time (many of them clearly labeled nonuniformly in male cells) inversely proportional to the drop in the H-only class of labeled interphases (compare Figures 5 and 6).

It is clear then that the DNA synthesis of heterochromatin here is out of phase with respect to that of euchromatin just as the condensation of heterochromatin, observed cytologically, is out of phase. Furthermore, when no heterochromatin is present, as in females, no out-of-phase DNA synthesis was detected.

Limitations of the experimental procedures: It seems reasonable to state that there is a late, asynchronous replication of H-DNA in *P. obscurus*. There are, however, a number of difficulties inherent in the procedures used which should be considered because they limit the precision that can be attained with autoradiographic technics in this material.

Some of the difficulties encountered are: (1) No label was ever observed in autoradiographic preparations until 1.5 hours after application of H^3TDR to the potato, and then only in a few of the large somatic interphases of a few (12%) individuals. (2) The mitotic index was variable but low. (3) The degree of synchrony of somatic cells could not be analyzed. (4) The proportion of non-dividing (endomitotic or permanent interphase) cells was high; the highest proportion of labeled cells recorded was at 4.5 hours fixation and even then less than 50% were labeled in a large sample of somatic, diploid abdominal cells. (5) The individual uptake of H^3TDR was variable, depending on the feeding intensity of the insect and also on the proportion of cells in S when the precursor was available, so that the length of the free pool time of the precursor (CRONKITE, BOND, FLIEDNER, and RUBINI 1959) could not be determined.

Cell-cycle estimations in relation to H-E asynchrony: In view of these difficulties, only rough measures of G, S, and D could be obtained. Nevertheless, it is not possible that the H-only and E-only labeled interphase classes which were observed are due to distribution of label to daughter nuclei during cell division following the application of H^3TDR . A cogent reason for saying this is that H-only and E-only labeled classes were first observed in the 3 hour fixations, at which time labeled mitoses were almost completely absent.

Asynchronies of DNA synthesis in other organisms: DNA asynchrony has been described in *Tradescantia* (WIMBER 1961), *Vicia* (WOODWARD, RASCH, and

SWIFT 1961) and Secale (LIMA-DE-FARIA 1959). In these cases, no differential genetic activity is known to be associated with the asynchrony.

However, heterochromatization, differential thymidine labeling, and genetic function seem to be emerging as related phenomena in studies on grasshopper spermatogenesis (LIMA-DE-FARIA 1959, 1961), hamster cells (TAYLOR 1960), and human material (BEUTLER, YEH, and FAIRBANKS 1962; MORISHIMA, GRUMBACH, and TAYLOR 1962). Similar relationships are probably operating in mice (LYON 1961).

The state of affairs in mealy bugs is straightforward once the salient features of the lecanoid genetic system are taken into account. This syndrome of chromosomal behavior is found in most mealy bugs and some related families (BROWN and NUR 1964). It is characterized by the fact that the paternal haploid set of chromosomes in the males becomes heterochromatic in early cleavage and persist in this condition throughout ontogeny. In males there is no pairing in meiotic prophase and the chromosomes separate equationally at metaphase I. The H set segregates from the maternal E set in anaphase II, which is the reduction division in the type of meiosis characteristic of the mealy bugs and their allies. Each H set degenerates post-meiotically, as a haploid nucleus, without undergoing spermiogenesis. Only the haploid E nuclei form mature sperm. All chromosomes in females and the maternal chromosomes in males are euchromatic throughout ontogeny. Meiosis is normal in females.

In the lecanoid system heterochromatization is facultative; the set of euchromatic chromosomes in a sperm either stays euchromatic or turns heterochromatic in the next generation in some unknown way dependent on the fate of the embryo, whether male or female.

In any case, males are characterized by the presence of a heterochromatic set of chromosomes. This H set is largely genetically inert. The evidence for this is threefold. (1) If a male has a normal allele on an H chromosome and a mutant "recessive" allele on the homologous E chromosome, his phenotype for that trait is mutant (BROWN and NUR 1964). (2) When X-ray induced dominant lethals are introduced into the heterochromatic set, via the sperm, no lethality of males ensues up to fairly high doses, but when dominant lethals are on the E set, the males die even at very much lower doses (BROWN and NELSON-REES 1961). (3) RNA synthesis in the H set is slow or absent (BERLOWITZ 1965).

One evidence for activity of the H set is that the H set of one species will not substitute for that of a related species (NUR and CHANDRA 1963).

Males surviving high-dosage paternal irradiation (30,000–120,000 rep), interestingly enough, are sterile so that some role in male fertility must be attributed to the heterochromatic set (NELSON-REES 1962). Furthermore these surviving males contain the entire bulk of H (95%) so that an approximately normal amount of H is necessary for male viability.

As an extension of the previously discussed systems where chromosomal asynchrony of DNA replication was found, it is clear that the late, asynchronous replication of DNA of one haploid set of chromosomes in male mealy bugs is associated with several functional characteristics: inertness with respect to Men-

delian genes, dominant lethals, and RNA synthesis; activity at least with respect to male fertility; lack of transmission to the next generation; and heterochromatization.

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SUMMARY

DNA synthesis in mealy bugs was studied by tritiated thymidine autoradiography, in order to determine whether there is a differential time of synthesis between the heterochromatic and euchromatic haploid sets of chromosomes. Cells labeled in only the heterochromatic and only the euchromatic sets were detected under various experimental conditions. On this evidence, it was concluded that the heterochromatic set synthesizes its DNA during roughly the last half of the synthetic period and the euchromatic set during roughly the first half. Uncertainties in the results of mitotic cycle experiments presented in the report and limitations in the methodology are discussed. The late, asynchronous replication of heterochromatin DNA in mealy bugs as associated with other genetic characteristics of heterochromatin in this system is discussed.

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