Differential Replication of the Chromosomes of *Pallavicinia longispina*, Liverwort

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Examples of differential replication of DNA in chromosomes are presented in many papers since the first findings of this phenomenon by Lima-de-Faria (1959). Among these studies the early replication of DNA in heterochromatin are reported in some animals by Hsu, Schmid and Stubblefield (1964), Ray-Chaudhuri and Singh (1972) and in higher plant by Tanaka (1965). The present author has also reported similar early replicating DNA in the heterochromatin of some species of liverworts (Tatuno, Tanaka and Masubuchi 1970, Masubuchi 1971, 1973, 1974).

In the present paper the differential replication of DNA among the euchromatic and heterochromatic chromosomes in *Pallavicinia longispina*, liverwort, will be reported.

Materials and methods

The female gametophytes of *Pallavicinia longispina* Mitt. collected from a woody slope along a stream in Mitaki-Chyo, Hiroshima City, were used for the present study. About 300 thalli of this species were grown on sanitary cotton moistened with tap-water in glass Petri dishes of 15 cm in diameter and left in an incubator at a temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, illuminated by a fluorescent light (300 Lux). Three to four weeks later, newly elongated thalli of about 0.5 to 1.0 cm in length were used for the present investigation. Methods in the preparation for the autoradiography were as follows:

³H-thymidine pulse label treatment: The thalli were immersed in 20 $\mu\text{Ci/ml}$ ³H-thymidine medium (TRA. 61, thymidine-6-T(n), specific activity 5000 mCi/mmol, obtained from Japan Radiochemical Centre) diluted with 1/2 Knop's solution at 22°C for 2 hours. The thalli were then washed thoroughly with a nonisotopic 1/2 Knop's solution containing an excess of unlabelled thymidine (25 $\mu\text{g/ml}$). A part of the thalli was fixed immediately and the rest were left on moistened cotton for further growth. Each 20 thalli was collected at various intervals (6, 8, 10, 15, 20, 25 and 30 hour) after the beginning of pulse labelling and fixed in a modified Carnoy's solution (ethanol: glacial acetic acid: chloroform = 3: 1: 1) at 10°C for 5 to 24 hours.

Preparation for autoradiography: After fixation, the thalli were stained by the Feulgen's nuclear reaction. This was accomplished by hydrolyzing the materials with 1N-HCl at 60°C for 13 minutes. After hydrolysis the materials were immersed in Schiff's reagent for at least 3 hours. Then, only the meristematic regions of the

thalli were squashed in 45% acetic acid under a cover slip on the slide glass coated with gelatin. After the removal of the cover slip by the dry ice method, Fuji stripping film ET-2F (Fuji Photofilm Co., Ltd.) was applied on the squashed material. Slides were air-dried and stored in a dark box. They were placed in a refrigerator at 4°C for 2 weeks for exposure. These slides for autoradiography were developed with Rendol (Fuji Photofilm Co., Ltd.) at 20°C for 6 minutes. The number of silver grains was counted in each preparation and its mean and standard deviation were calculated. To make a more detailed observation of the morphological characteristics of the nuclei, the silver grains were removed using the solution containing one part of 13% sodium thiosulphate and one part of 10% potassium ferricyanide.

Results

1. *Morphology of nuclei during the mitotic cell cycle*

Meristematic regions in the apex of thalli contained dividing cells of each stage of the mitotic cell cycle. Morphological features of interphase nuclei and mitotic chromosomes were observed in the squashed preparation stained by Feulgen's nuclear reaction.

A. *Interphase nuclei*

The interphase nuclei varied in size and in shape of chromatin. In most interphase nuclei, the chromatin was observed forming a large condensed body. The shape of the condensed body was globose with a diameter varying from 5 to 7 μm . In some nuclei the condensed body was club-shaped or rod-shaped with irregular surface. The interphase nuclei can be classified into four types by the shape of chromatin and by the size of nuclei.

1) Daughter cell nucleus at the stage immediately after telophase. The size of nucleus is the smallest among the meristematic nuclei. One club- or rod-shaped condensed body of chromatin occurs in each nucleus (Fig. 1A).

2) Diffused nucleus. All of the chromatin are entirely of the extended state (Fig. 1B).

3) Partially condensed nucleus. The nucleus has a large condensed body whose shape is globose (Fig. 1C).

4) Fully condensed nucleus. The nucleus has a large condensed body of club- or rod-shape (Fig. 1D).

The size of the nucleus of the latter three types is similar to each other and about twice as large as that of daughter nucleus in diameter.

B. *Mitotic chromosomes*

The chromosome number of this species was observed to be $n=8$. This chromosome number agrees with that of Heitz (1928), Tatuno (1933, 1941) and Segawa (1965).

At prophase, one of the median size chromosome was stained deeply, while the rest of the chromosomes stained slightly (Fig. 1E). In the present observation the deeply stained chromosome was identified to be the X chromosome of this species,

Fig. 1. Morphology of the nuclei of the female gametophyte of *Pallavicinia longispina* ($n=8$) at each phase during the mitotic cell cycle. Arrows indicate the X chromosomes. A–D, interphase nuclei. E–I, mitotic chromosomes. A, daughter cell nuclei immediately after telophase. B, diffused nucleus. C, partially condensed nucleus. D, fully condensed nucleus. E, prophase chromosomes. F, prometaphase chromosomes. G, metaphase chromosomes. H, anaphase chromosomes. I, telophase chromosomes. The bar is equal to 10 μm .

because it consists of similar heterochromatin pattern in most regions of both arms to those of the observation of Segawa (1965). Thus, most part of the large condensed bodies observed in interphase nuclei are recognized as being the X chromosome (Fig. 1A-D).

At prometaphase, each chromosome of the complement exhibited respective

Fig. 2. Serial alignments of somatic chromosomes of the female gametophyte of *Pallavicinia longispina* ($n=8$) at prometaphase. A, photograph. B, sketch. The bar is equal to 10 μm .

Table 1. Measurement of somatic chromosomes of the female gametophyte of *Pallavicinia longispina* ($n=8$) at prometaphase

Chromosome no.	Length in μm			Form (%)
	Short arm	Long arm	Total	
1 (X)	2.4 (2.4)*	5.0 (5.0)	7.4 (7.4)	32.4
2	6.0	7.7	12.7	47.2
3	4.6 (1.1)	6.9	11.5 (1.1)	40.0
4	2.4 (0.4)	7.9	10.3 (0.4)	23.3
5	3.1 (0.4)	6.5	9.6 (0.4)	32.3
6	3.9	4.8	8.7	44.8
7	3.2 (0.7)	4.7	7.9 (0.7)	40.5
8	1.8	4.8	6.6	27.3

* Numbers in () indicate heterochromatic regions.

morphological features (Fig. 1F and Fig. 2). Morphological distinction of chromosomes was analysed in the prometaphase chromosomes. Results of the measurement of the length of prometaphase chromosomes are shown in Table 1. Eight chromosomes can be divided into one X chromosome (chromosome 1) and seven autosomes which are numbered from 2 to 8 by the order of length. X chromosome was stained deeply consisting of heterochromatin in most parts of both arms. The

four chromosomes, 3, 4, 5 and 7, were euchromatic, except the small heterochromatic segments situated in the distal position of the short arms. The three chromosomes, 2, 6 and 8, were totally euchromatic.

At metaphase, all of the eight chromosomes were stained deeply (Fig. 1G). Each chromosome can be distinguished from each other by the length and by the position of the centromere. The length of chromosomes vary gradually showing a homogeneous karyotype. The position of the centromere is median or submedian in all of the chromosomes, thus showing a symmetric karyotype.

At anaphase, each chromatid was stained uniformly (Fig. 1H). At telophase, chromatids form a densely packed group at two poles, and then begin to diffuse (Fig. 1I).

C. Number of cells at each phase in the mitotic cell cycle

As mentioned above, the cells of each phase in the mitotic cell cycle can be easily distinguished by the size of the nuclei and by the condensed pattern of the chromatin.

Table 2. Number and frequency of the nuclei of each phase in mitotic cell cycle in the meristematic region of the female gametophyte of *Pallavicinia longispina* (n=8)

Interphase				
	Daughter cell nucleus	Diffused nucleus	Partially condensed nucleus	Fully condensed nucleus
Number and percentage	354 12.5%	218 7.7%	1224 43.1%	648 22.8%
	2444 86.1%			
Prophase	Prometaphase	Metaphase	Ana-, Telophase	Total
113 4.0%	102 3.6%	47 1.7%	132 4.7%	2838

The proportion of the cells in the different phases was calculated from 2838 cells in the meristematic regions of thalli. The results obtained are shown in Table 2. Nuclei at interphase appeared with the highest frequency, 86.1%, while those at metaphase appeared with the lowest frequency, 1.7%. At interphase, partially condensed nuclei showed the highest frequency, and the diffused nuclei the lowest and in the nuclei of mitotic phase, ana-telophase nuclei the highest, and metaphase nuclei the lowest.

2. Duration of the mitotic cell cycle

The numbers of ³H-thymidine labelled and unlabelled metaphase nuclei were scored in the cells fixed at 2 to 30 hour after the beginning of 2 hours pulse labelling by ³H-thymidine. The results are given in Table 3 and Fig. 3.

The first appearance of the labelled metaphase was observed at 6 hour after the beginning of pulse labelling. The percentage of labelled metaphase was 7.5% in

proportion to unlabelled metaphase. A maximum percentage of labelled metaphase was 79.7% in the cell population fixed at 15 hour after the beginning of pulse labelling. Duration of one mitotic cell cycle (tC) was estimated as the distance, measured at the 50 percent intercept, between the first and second ascending curves, which is $tC = 36.0 - 7.5 \div 28.5$ hours. The average length of the duration of S period (tS) can be calculated as the distance, measured at the 50 percent intercept, between the first ascending and the first descending curves, which is $tS = 22.0 - 7.5 \div 14.5$ hours. In a

Table 3. Number of labelled and unlabelled metaphase nuclei in the meristematic cells of the female gametophyte of *Pallavicinia longispina* ($n=8$) after the beginning of 3H -thymidine pulse labelling

Fixation time after the beginning of 3H -thymidine pulse labelling (hours)	Number of nuclei observed	Number of labelled nuclei	Number of unlabelled nuclei	Percent of labelled nuclei
2	102	0	102	0.0
6	53	4	49	7.5
8	111	64	47	57.7
10	124	80	44	64.5
15	79	63	16	79.7
20	44	27	17	61.4
25	78	24	54	30.8
30	131	60	71	45.8

Fig. 3. Proportion of labelled metaphase nuclei at various times after the beginning of 3H -thymidine pulse labelling in the female gametophyte of *Pallavicinia longispina* ($n=8$).

similar way the average length of the duration of G_2 phase (tG_2) + prophase (tP) can be estimated as the distance between the time of the beginning of pulse labelling and the intersection of 50 percent abscissa of the ascending portion of the curve, which is 7.5 hours. Then, the average length of the duration of G_1 phase (tG_1) + metaphase (tM) + ana-telophase ($tA-T$) can be estimated by applying the following formula: $tG_1 + tM + tA - T = tC - (tG_2 + tP + tS)$, which is $28.5 - (17.5 + 4.5) \div 6.5$ hours.

The duration of each phase in the mitotic stage was estimated by the ratios between number of cells in each phase and duration of S period (Sparvoli *et al.* 1966). The number of cells in each phase was counted in the meristematic regions. The results are shown in Table 2. The duration of the mitotic stage was calculated by the formula,

$$\frac{2838}{tC} = \frac{2838}{28.5} = \frac{2444}{tI} = \frac{116}{tP} = \frac{102}{tPM} = \frac{47}{tM} = \frac{129}{tA-T}$$

The duration of each phase was calculated as follows: Interphase $\div 24.5$ hours, prophase $\div 1.2$ hours, prometaphase $\div 1.0$ hour, metaphase = 0.5 hour, ana-telo-

phase ± 1.3 hours. The results calculated by this method agreed with those obtained from the frequency curve of labelled metaphase.

Fig. 4. Autoradiographs of three types of labelled interphase nuclei observed immediately after the ^3H -thymidine pulse labelling in the female gametophyte of *Pallavicinia longispina* ($n=8$). A, B, diffused nucleus. C, D, partially condensed nucleus, showing the labelling only over the diffused chromatin. E, F, partially condensed nucleus, showing the labelling over the diffused chromatin and condensed chromatin. B, D and F are the photographs of the nuclei shown in A, C and E, respectively after the removal of the silver grains. The bar is equal to 10 μm .

3. Autoradiography of interphase nuclei

Among the interphase nuclei fixed at immediately after the 2 hours pulse labelling, only the diffused nuclei and partially condensed nuclei were labelled. Therefore, it can be considered that the two nuclei are to be at S period, while the daughter

cell nuclei and the fully condensed nuclei are to be in other stages of interphase, probably the former to be G_1 period and the latter to be G_2 period. Thus, each duration of interphase nuclei was calculated by the formula,

$$\frac{2444}{tI} = \frac{2444}{2.45} = \frac{354}{tG_1} = \frac{1442}{tS} = \frac{648}{tG_2}$$

The results were as follows: G_1 period $\doteq 3.5$ hours, S period $\doteq 14.5$ hours, G_2 period $\doteq 6.5$ hours.

The distribution of silver grains over the chromatin in interphase nuclei was investigated in the labelled interphase nuclei fixed at immediately after the pulse labelling. The silver grains occurred mainly over the diffused chromatin and rarely over the condensed chromatin. When the silver grains do appear over the condensed chromatin, they are observed on the peripheral regions. By the labelling pattern, the nuclei of S period can be divided into three types:

Type A. Diffused nucleus, labelled (Fig. 4A, B).

Type B. Partially condensed nucleus labelled in only diffused chromatin (Fig. 4C, D).

Type C. Partially condensed nucleus labelled in both diffused and condensed chromatin (Fig. 4E, F).

Table 4. Frequency of the three types of labelled interphase nuclei in the female gametophyte of *Pallavicinia longispina* (n=8)

Fixation time after the beginning of pulse labelling (hour)	Labelled interphase nuclei observed	Diffused nuclei	Partially condensed nuclei	
			with unlabelled condensed chromatin	with labelled condensed chromatin
2	300	165 (55.0%)	127 (42.3%)	8 (2.7%)
6	300	66 (22.0%)	153 (51.0%)	81 (27.0%)
8	300	20 (6.7%)	94 (31.3%)	186 (62.0%)
10	300	9 (3.0%)	64 (21.3%)	227 (75.7%)

The occurrence of the three types of nuclei was counted in each of the thalli fixed at 2, 6, 8 and 10 hour after the beginning of pulse labelling and the results are shown in Table 4 and Fig. 5. At 2 hour, the nuclei which belongs to Type A showed the highest frequency (55.0%) among the labelled nuclei. Among the three types of nuclei, the nuclei of Type C showed the most numerous silver grains over its chromatin. At this time, it was found that in all of the DNA synthesizing nuclei the labelling occurred mainly in the diffused chromatin. At 8 hour, the frequency of the nuclei of Type C increased markedly (62.0%), while that of the nuclei of Type A and Type B decreased.

Presumably, the changes in the frequency of labelled interphase nuclei are due to the successive modification of the condensation of chromatin during S period. That is, Type A is considered to be the nucleus at early S period because its frequency

decreased with lapse of time. In Type A, both the heterochromatin and euchromatin are at the diffused state. Type B is the nucleus at early to middle S period, where condensation of the heterochromatin is still taking place after DNA synthesis, and the chromatin of the peripheral regions of condensed chromatin is synthesizing DNA. Type C is considered to be the nucleus at middle to late S period, because its number has increased with lapse of time. In the nuclei of Type C silver grains are observed over the condensed chromatin. The occurrence of silver grains over the condensed chromatin in later fixation time indicates that the DNA of this chromatin, i. e. heterochromatin, has been synthesized during the diffused state.

From these observations, it can be concluded that the chromatin synthesizes its DNA in the diffused state regardless of being either heterochromatin or euchromatin. Since the condensed bodies are labelled during 2 hours pulse labelling, a part of the heterochromatin is considered to condense successively to the DNA replication.

Fig. 5. Frequency of occurrence of the three types of labelled interphase nuclei after the beginning of ^3H -thymidine pulse labelling in the female gametophyte of *Pallavicinia longispina* ($n=8$). ●---● diffused nuclei, ●---● partially condensed nuclei with unlabelled condensed chromatin, ●—● partially condensed nuclei with labelled condensed chromatin.

4. DNA synthesis in the heterochromatin and euchromatin

In the cells fixed at 2 to 25 hour after the beginning of 2 hours pulse labelling, the number and distribution of the silver grains over the heterochromatic regions or euchromatic regions of prometaphase chromosomes were investigated. The results are shown in Fig. 6 and Table 5. The changes of labelling patterns in the chromosomes at each time interval after pulse labelling are shown in Fig. 7. Labelling patterns of the individual chromosomes observed commonly in each time interval are illustrated in Fig. 8.

The first appearance of the silver grains over the prometaphase chromosomes was observed at 6 hour, when the grains occurred only over the euchromatic regions in autosomes. At 8 hour, most of the silver grains occurred over the euchromatic regions. The silver grains over the heterochromatic regions were observed in only two chromosome complements among the six chromosome complements studied at this time, and its number was only three. These silver grains were found to occur over the autosomes and not over the X chromosome.

The average number and its standard deviation of silver grains over the euchromatic regions at 8 hour was 56.5 ± 7.6 , while that over the heterochromatic regions was 1.4 ± 0.3 . The labelling pattern of individual chromosomes which appeared commonly at 8 hour fixation time was as follows: X chromosome was observed without

Fig. 6. Autoradiographs of the prometaphase chromosomes of the female gametophyte of *Pal-lavicinia longispina* ($n=8$) fixed at each time interval after the ^3H -thymidine pulse labelling. Arrows indicate the X chromosomes. A, prometaphase chromosomes fixed immediately after the pulse labelling. B, C, D, E and F are fixed at 8, 10, 15, 20 and 25 hour after the beginning of pulse labelling, respectively. The bar is equal to $10\ \mu\text{m}$.

labelling. In chromosome 2, euchromatic regions in the distal and subdistal positions of the short arm and those of the middle position of the long arm were labelled. In chromosome 3, most of the euchromatic regions were labelled except those of the distal position of the long arm, while the heterochromatic regions of the distal position of the short arm were not labelled. In chromosome 4, euchromatic regions in

the middle and distal positions of the long arm were labelled, while the short arm having heterochromatic regions was not labelled. In chromosome 5, euchromatic regions in the proximal, middle and subdistal positions of the long arm were labelled, while the short arm having heterochromatic region was not labelled. In chromosome 6, euchromatic regions in the distal and proximal positions of both arms were labelled. In chromosome 7, euchromatic regions in the middle and proximal positions of the short arm were labelled, while heterochromatic region of the short arm and the euchromatic region of the long arm were not labelled. In chromosome 8, euchromatic regions in the distal positions of both arms and middle and proximal positions of the short arm were labelled.

Table 5. Mean grain number and its standard deviation over heterochromatin and euchromatin in the prometaphase chromosomes of the female gametophyte of *Pallavicinia longispina* (n=8) fixed at each time interval after the beginning of ^3H -thymidine pulse labelling

Chromosome no.	Kinds of chromatin	Number of silver grains				
		8h	10h	15h	20h	25h
1 (X)	H*	0.3 ± 0.3	0.5 ± 0.3	5.0 ± 1.4	15.5 ± 2.5	5.7 ± 1.3
	E**	—	—	—	—	—
2	H	—	—	—	—	—
	E	11.8 ± 1.8	11.4 ± 2.2	6.2 ± 1.3	5.0 ± 1.0	7.8 ± 1.7
3	H	1.0 ± 0.2	0.1 ± 0.1	1.0 ± 0.5	1.5 ± 0.5	2.7 ± 1.0
	E	11.0 ± 2.0	10.6 ± 2.1	7.4 ± 1.9	5.5 ± 0.5	3.8 ± 1.5
4	H	0.1 ± 0.2	0.3 ± 0.2	1.2 ± 0.5	1.5 ± 0.5	1.7 ± 0.6
	E	5.7 ± 0.8	6.8 ± 1.3	5.4 ± 1.5	4.0 ± 1.0	2.8 ± 1.1
5	H	0.2 ± 0.1	0.4 ± 0.2	0.8 ± 0.4	2.5 ± 0.5	2.5 ± 0.9
	E	8.2 ± 1.7	8.4 ± 1.5	5.2 ± 1.7	9.0 ± 3.0	3.0 ± 1.8
6	H	—	—	—	—	—
	E	8.0 ± 0.6	11.1 ± 2.2	5.0 ± 1.2	5.0 ± 1.0	4.2 ± 1.2
7	H	0.2 ± 0.2	0.3 ± 0.2	2.2 ± 0.7	1.0 ± 0.1	1.7 ± 0.8
	E	7.2 ± 1.4	6.8 ± 1.2	2.8 ± 1.2	2.0 ± 0.2	2.2 ± 1.1
8	H	—	—	—	—	—
	E	4.7 ± 1.1	5.8 ± 1.0	3.2 ± 0.7	6.0 ± 2.0	3.5 ± 1.1
one chromosome complement	H	1.4 ± 0.3	1.5 ± 0.7	10.2 ± 2.7	22.0 ± 1.0	14.2 ± 3.2
	E	56.5 ± 7.6	60.8 ± 10.2	35.2 ± 6.3	36.5 ± 6.5	26.8 ± 8.5

* is heterochromatin, ** is euchromatin.

At 10 hour after the beginning of pulse labelling, the number of silver grains which occurred over the whole heterochromatic and euchromatic regions per one prometaphase chromosome complement was 1.5 ± 0.7 and 60.8 ± 10.2 , respectively. Therefore, almost all of the DNA in the heterochromatin is not synthesized in the preceding 10 hour, when the nuclei were in the last stage of S period. The prometaphase chromosome complement which was observed to be a common labelling pattern is illustrated in Figs. 6C and 8–10h. In the 10 hour chromosome complement X chromosome was observed not to be labelled even to this time. In chromosome 2,

euchromatic regions except the proximal and middle positions of the long arm were labelled. In chromosome 3, euchromatic regions in the middle position of the short arm and middle and distal positions of the long arm were labelled. Euchromatic region of the long arm was observed to be labelled heavily. In chromosome 4, euchromatic proximal regions of both arms, subdistal region of the short arm and distal region of the long arm were labelled. In chromosome 5, euchromatic middle and proximal regions of the short arm and proximal and distal regions of the long arm were labelled. In chromosome 6, euchromatic regions of both arms were labelled totally. Its distal position of the short arm and middle position of the long arm were labelled heavily. In chromosome 7, the whole regions except the hetero-

Fig. 7. Mean grain number and its standard deviation over heterochromatin (●—●) and euchromatin (●---●) in the female gametophyte of *Pallavicinia longispina* ($n=8$) after the beginning of ^3H -thymidine pulse labelling.

chromatic region in the distal position of the short arm were labelled. In chromosome 8, euchromatic regions in the middle and proximal positions of the short arm and in the proximal and subdistal positions of the long arm were labelled. The proximal regions of both arms were labelled heavily.

At 15 hour after the beginning of pulse labelling, the heterochromatic regions were found to be labelled for the first time and the total number of silver grains over them was 10.2 ± 2.7 . In the preparation of 15 hour the euchromatic regions of autosomes were observed to be labelled, though the total number of silver grains over them was 35.2 ± 6.3 showing a decrease in number compared with those of the euchromatic regions appeared at 8 and 10 hour. A common labelling pattern of chromosome complements is given in Figs. 6D and 8–15h. In X chromosome,

heterochromatic regions in the distal position of the short arm and in the proximal and middle positions of the long arm were observed to be labelled. The heterochromatic region in the distal position of its short arm was labelled heavily. In chromosome 2, all of the euchromatic regions were labelled uniformly, except the distal region of the long arm which is euchromatic. In chromosome 3, euchromatic proximal region of the short arm and all of the long arm were labelled, while the heterochromatic region located in the distal position of the short arm were not labelled. In chromosome 4, the euchromatic proximal regions of both arms and an euchromatic region in the middle position of the long arm were labelled, while the heterochromatic region located in the distal position of the short arm was not labelled. In chromosome 5, all of the regions of the chromosome were labelled, except the distal heterochromatic region in the short arm and distal euchromatic one in the long arm which appeared unlabelled. In chromosome 6, euchromatic distal regions of both arms appeared without label, while the rest of the chromosome segments appeared to be labelled. In chromosome 7, the heterochromatic region located in the distal regions of the short arm and the euchromatic regions in the middle positions of both arms were labelled. In chromosome 8, the euchromatic regions in the proximal and distal positions of the long arm were labelled.

At 20 hour after the beginning of pulse labelling, a larger number of silver grains over the heterochromatic regions, 22.0 ± 1.0 per one chromosome complement, was counted in comparison with those at 15 hour. The heterochromatic regions of X chromosome had marked by heavy labelling at 20 hour. The number of silver grains on the X chromosome was the highest and was counted to be 15.5 ± 2.5 . The number of the silver grains on the euchromatic regions, on the other hand, was counted to be 36.5 ± 6.5 per one chromosome complement, a decreased number compared with those at 8 and 10 hour. One of the labelling patterns in 20 hour is given in Figs. 6E and 8–20h. Almost all the regions of X chromosome were observed to be labelled. Its subdistal regions of the long arm was observed to be labelled most heavily than the other regions. In chromosome 2, euchromatic proximal regions of both arms and distal regions of the long arm were labelled slightly. In chromosome 3, the heterochromatic region in the distal position of the short arm and euchromatic regions in the middle and distal positions of the long arm were labelled slightly. In chromosome 4, all parts of chromosome were labelled slightly. In chromosome 5, the heterochromatic region in the distal position of the short arm were labelled heavily, while the euchromatic region in the distal position of the long arm was not labelled. In chromosome 6, euchromatic regions in the middle positions of both arms and in the distal position of the long arm were labelled slightly. Chromosome 7 was labelled very slightly showing the weakest labelling among eight chromosomes. The heterochromatic distal position of the short arm and euchromatic middle position of the long arm were labelled. In chromosome 8, euchromatic proximal regions of both arms and those in the middle and distal positions of the long arm were labelled slightly.

At 25 hour after the beginning of pulse labelling, the total number of silver grains per one chromosome complement was observed to be very low as 41.0 ± 11.7 . The number of silver grains over the euchromatic regions of a chromosome complement

was 26.8 ± 8.5 , while that over the heterochromatic regions was 14.2 ± 3.2 . Therefore, both of the heterochromatic regions and euchromatic regions were found to synthesize DNA 25 hours before fixation, when the cells are presumed to be at early

Fig. 8. Labelling patterns commonly occurred in the prometaphase chromosomes of the female gametophyte of *Pallavicinia longispina* ($n=8$) fixed at each time interval after the beginning of ^3H -thymidine pulse labelling. Grains at 8h and 10h show the DNA replicating regions in the last S phase (LS). Grains at 2h, 15h and 20h–25h show the DNA replicating regions in the G_2 period (G_2), middle S period (MS) and early S period (ES), respectively. The bar is equal 10 μm .

S phase. The chromosome complement with common labelling pattern is given in Figs. 6F and 8–25h. In X chromosome, heterochromatic regions in the distal positions of both arms, especially of the long arm, were labelled heavily. The remaining regions of both arms were labelled slightly. In chromosome 2, euchromatic

regions in the distal and proximal positions of both arms were labelled. In chromosome 3, the distal positions of both arms, especially heterochromatic region of the short arm were labelled. In the chromosome 4, the heterochromatic region located in the distal position of the short arm and euchromatic regions in the middle and subdistal positions of the long arm were labelled. In chromosome 5, the heterochromatic region located in the distal position of the short arm and euchromatic regions in the middle position of the short arm and in the proximal position of the long arm were labelled. In chromosome 6, euchromatic regions in the proximal positions of both arms and in the distal position of the long arm were labelled. In

Fig. 9. Changes of labelling pattern of chromosomes X, 2 and 3 at each time interval from 2 to 25 hour after the beginning of ^3H -thymidine pulse labelling. Black portions of chromosomes show heterochromatic regions and white, euchromatic regions. LS, MS and ES represent the period when each chromosome incorporated ^3H -thymidine at late S, middle S and early S, respectively.

chromosome 7, the heterochromatic regions located in the distal position of the short arm and euchromatic region in the distal position of the long arm were labelled slightly. In chromosome 8, euchromatic regions in the middle positions of both arms and in the distal position of the long arm were labelled slightly.

The timing of the chromosomal DNA replication in the female gametophyte of *Pallavicinia longispina* ($n=8$) is summarized schematically in Fig. 9. As described in above paragraphs, the appearance of the silver grains over the mitotic prometaphase chromosomes were observed for the first time at 6 hour after the beginning of

pulse labelling. In these prometaphase chromosomes the silver grains appeared only over euchromatic autosomes and not over heterochromatic X chromosome. At 8 and 10 hour, almost all of the silver grains were still observed only over the euchromatic regions of prometaphase chromosomes. The appearance of silver grains over the heterochromatic regions was observed for the first time at 15 hour after the beginning of pulse labelling. These observations suggest that the DNA synthesis in heterochromatin had completed at 15 hours before the formation of the shape of prometaphase chromosomes. The time of 15 hours before the mitotic stage at prometaphase is calculated to correspond to the 4 to 12 hours before the end of the S period, which is the early to middle stage of the S period. Therefore, the heterochromatin of which most parts are composed of X chromosome, is an early DNA replicating one.

The silver grains over the euchromatic regions of prometaphase chromosomes were observed at all fixation times after the beginning of pulse labelling. This observation indicates that the synthesis of DNA in euchromatin takes place throughout the S period.

Discussion

The phenomenon of early DNA replication in heterochromatin was originally found by Hsu, Schmid and Stubblefield (1964) in *Microtus oregoni* and by Tanaka (1965) in *Spiranthes sinensis*. The same phenomenon has been observed in some species of liverworts such as *Pellia neesiana* (Tatuno, Tanaka and Masubuchi 1970, Masubuchi 1971) *Pallavicinia lyellii*, *Plagiochila japonica* (Masubuchi 1973), *P. ovalifolia* (Masubuchi 1974) and some species of snakes such as *Boiga trigonata*, *B. forsteni*, *Naja naja*, *Vipera resseli tusselli* (Ray-Chaudhuri and Singh 1972). Moreover there are some additional species which have early replicating heterochromatin, though unpublished by the present author, such as *Riccardia pinguis*, *Trichocolea tomentella*, *Frullania japonica* and *Fossombronina akiensis*. These results obtained in liverworts and snakes clearly prove the existence of early DNA replicating heterochromatin.

On the other hand, there are some reports which suggest that timing of DNA replication of chromatin is changeable. That is, Mukherjee and Gosal (1969) demonstrated in Golden hamster that the Y chromosome shift from late to early replication during the process of spermatogonial maturation. In rats, Bianchi and Bianchi (1969) reported that the shift of replication in Y chromosome occurs in the first division of the fertilized eggs. Furthermore, Ananiev and Gvozdev (1974) found that the timing of the replication of translocated heterochromatic and euchromatic regions is changeable by using special enhancers of position-effect of polytene chromosomes in *Drosophila melanogaster* and ^3H -thymidine autoradiography. In the present paper, although both the autosomal and sex chromosomal heterochromatins exhibit a nearly synchronous replication behaviour, it is found that the heterochromatic regions situated in chromosomes 3, 4 and 5 complete replication slightly earlier than those situated in other chromosomes. This apparent variability in the replication of heterochromatin suggests that late replication (Brown 1966. Lima-de-

Faria and Jaworska 1968, Lima-de-Faria 1969) may not be the basic characteristic of heterochromatin replication.

What is the difference between heterochromatin and euchromatin? In *Pallavicinia longispina* reported in the present paper, heterochromatin completes its DNA replication by at least about 4 hours before the end of S period, while DNA replication of euchromatin takes place throughout the S period. This observation indicates that the heterochromatin must complete its DNA replication in a shorter period of time than euchromatin. The same phenomenon was observed in *Plagiochila ovalifolia* and in other species of liverworts which have early DNA replicating heterochromatin (Tatuno, Tanaka and Masubuchi 1970, Masubuchi 1971, 1973, 1974). For this phenomenon, Comings (1970) accounted that the heterochromatin replication may be the tendency of its replicons to replicate in greater unison than

Fig. 10. Schematic representation of the interrelationship between interphase nuclei and the labelling pattern of prometaphase chromosomes in the mitotic cell cycle of *Pallavicinia longispina*. The dots along the prometaphase chromosomes represent the labelling pattern at each time interval in heterochromatic X chromosome (black) and in euchromatic autosomes (white).

those in euchromatin. In the present investigation though no data could be mentioned such as the change of the number of replicating units (Tanaka 1969), the tendency that the duration of the replication of heterochromatin was significantly less than that of euchromatin was clearly recognized. This seems to be the most general characteristic of heterochromatin replication since this phenomenon occurs irrespective of species which have late or early replicating heterochromatin.

In the present material the number of diffused nuclei of which all chromatins are entirely of the extended state is very low (Table 2). This result suggests that the duration of diffused nuclei is very short. In the present investigation, four types of interphase nuclei were recognized. And from the sequence of DNA replication, it is identified that the daughter cell nuclei are to be G₂ period, diffused nuclei are to be early S period, partially condensed nuclei are to be from middle to late S period and

fully condensed nuclei are to be G_2 period. These results suggest that the heterochromatin forms condensed body at G_2 period and turns immediately into a diffused state at the beginning of S period and starts to replicate its DNA. During the middle S period, this heterochromatin condenses gradually after the completion of DNA replication and begins to form a condensed body from late S period to G_2 period. This condensed heterochromatic body shifts to the mitotic period without changing its structure (Fig. 10). This phenomenon was observed in *Pellia neesiana* (Tatuno, Tanaka and Masubuchi 1970, Masubuchi 1971), *Pallavicinia lyellii*, *Plagiochila japonica* (Masubuchi 1973), *P. ovalifolia* (Masubuchi 1974), *Frullania japonica*, *Riccardia pinguis*, *Trichocolea tomentella*, *Fossombronina akiensis* (unpublished data).

On the other hand, Tatuno, Tanaka and Yonezawa (1971) reported a different phenomenon. That is, the heterochromatin of *Calobryum rotundifolium*, liverwort, sets at early S period in a condensed state and begins to diffuse from middle to late S period. DNA replication in the heterochromatin of this species starts when heterochromatin begins to diffuse. The condensation of the heterochromatin occurs at the G_2 period. The same phenomenon was observed in *Pellia fabbronia* (unpublished data). The two species are found to have late DNA replicating heterochromatin. It seems that the timing of DNA replication in heterochromatin relates to the timing of dispersion and condensation of heterochromatin during S period.

Résumé

1. The estimated duration of the mitotic cell cycle of meristematic cells in the female gametophyte of *Pallavicinia longispina* was follows:

(period)	(hour)
G_1	3.5
S	14.5
G_2	6.5
M	4.0
<div style="display: flex; align-items: center; justify-content: center;"> <div style="font-size: 3em; margin-right: 10px;">{</div> <div> prophase 1.2 prometaphase 1.0 metaphase 0.5 ana-telophase 1.3 </div> </div>	
total cell cyte	28.5

2. The first appearance of silver grains over the mitotic prometaphase chromosomes was at 6 hour after the beginning of 2 hours pulse labelling. At this hour silver grains were only over the euchromatic regions, and not over the heterochromatic regions. This labelling pattern was also observed at 8 to 10 hour. The heterochromatic regions having silver grains were observed for the first time at 15 hour when euchromatic regions were also labelled. At 20 to 25 hour, the heterochromatic regions have more grains than the euchromatic regions. These results show that the duration of DNA replication in heterochromatin is shorter than that of euchromatin and that the heterochromatin completes its DNA replication earlier than euchromatin.

3. Differential replication of DNA is found among chromosomes in a complement: X chromosome completes its DNA replication earlier than any other autosomes; individual autosomes vary in the distribution of early replicating segments.

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