Meiosis in *Ornithogalum virens* (Liliaceae)

III. Pattern of RNA synthesis during meiotic prophase

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The incorporation of labeled precursors into RNA during male meiosis has been studied in various species of animals (Henderson 1964, Monesi 1965, Das *et al.* 1965, Utakoji 1966, and Brasiello 1968), and plants (Taylor 1958 1959, Hotta and Stern 1963, Das 1965, Sauter 1968). These studies indicate that the rates of RNA synthesis fluctuate depending on the stage of meiosis. Generally, the nucleolus disappears or becomes very small in early meiotic prophase in male animals, but in pollen mother cells of higher plants it is often very prominent. Little is known about the pattern of nucleolar RNA synthesis during plant pollen mother cell meiosis because of the difficulty of doing short term labeling experiments with tritium labeled compounds. The cytophotomeric work of Lin (1955) however, would indicate that in maize there is a doubling of RNA in mid meiotic prophase and a substantial increase in RNA per nucleolus in pachytene. In contrast, Das (1965) reported that little or no nucleolar RNA synthesis occurs in the very large nucleoli found in maize microsporocytes. Das immersed maize anthers in a solution of H^3 -uridine and observed the pattern of incorporation autoradiographically. We have utilized a technique where inflorescences of *Ornithogalum virens* were labeled *in vivo* using a wick feeding process (Gear and Spencer 1963, Church and Wimber 1969). The aims of this autoradiographic study were to observe: 1) the general overall pattern of RNA synthesis and 2) the pattern of nucleolar RNA synthesis during meiotic prophase. In addition, the cytological effects of an RNA synthesis inhibitor, actinomycin D, on meiosis *in vivo* were investigated.

Methods and materials

Autoradiography

O. virens has a racemous, indeterminant inflorescence on which all stages of meiosis can be found. Pre-meiotic cells and early stages of meiosis are in buds located near the tip of the inflorescence and successively later stages of meiosis occupy buds in proximal regions.

The plants were maintained at 18°C with continuous illumination before and during these experiments. H^3 -uridine (100 μ C/ml, 16.5 C/Mm, New England Nuclear) was supplied to the meiotic cells through a wick of cotton string threaded through the peduncle at the base of the inflorescence. Inflorescences were removed from the plant and fixed in 3:1 (100% ethanol to glacial acetic acid) after 2, 4, and 10

hours of labeling. Squash preparations (anthers from 1 bud per slide) were made from each inflorescence spanning all stages of meiosis. All preparations were treated with 2% perchloric acid for 40 minutes at 4°C to remove acid soluble RNA precursors. One series of slides from each of the timed samples was treated with 0.1% RNAase for 2 hours. All preparations were dipped in Kodak NTB liquid emulsion. The 2 and 4 hour samples were exposed for 3 weeks and the 10 hour sample for 8 days prior to processing for autoradiography. The autoradiographs were stained through the emulsion with Delafield's hematoxylin. Counts were made of silver grains overlying the chromosomes, nucleolus and cytoplasm.

Actinomycin D

Young inflorescences in which meiotic development of the older buds had progressed to the tetrad stage of meiosis were given actinomycin D (50 µg/ml) using the wick feeding technique. Control inflorescences were given distilled water by the same method. Inflorescences were sampled at various intervals 4 to 48 hours after the beginning of the wick feeding procedure. Inflorescences were fixed as before, stored in 70% ethanol, and a series of aceto-orcein squash preparations were made from each inflorescence spanning all stages of meiosis.

Results

Autoradiography

The earlier stages of meiotic prophase in *O. virens* are difficult to analyze. Following pre-meiotic interphase the chromosomes form a tight ball and are deeply staining. In most preparations the nucleolus is obvious. As prophase progresses the nucleus enlarges and the chromosomes are more loosely associated. Pachytene can easily be identified. For purposes of grain counting the cells were divided into four cytological groups: 1) pre-meiotic interphase 2) pre-pachytene 3) pachytene 4) diplotene. Cells within the anthers of a single bud are not always synchronous in meiotic development. Two and sometimes 3 relatively short, adjacent stages often occupy the same bud (i.e. diplotene, metaphase I and anaphase I).

Control slides treated with RNAase indicated that substantially all silver grains represented newly synthesized RNA. Counts were made of silver grains over the nucleolus, chromosomes and cytoplasm of all stages of meiosis in all timed samples. The uptake of H³-uridine was variable between inflorescences and between buds of the same inflorescence. However, as far as we could judge, availability within a bud appeared to be uniform, therefore rates of RNA synthesis as judged by grain counts were compared between stages that occupied the same bud. The grain count comparison was made on buds that had been labeled with H³-uridine for 2 hours (Table 1). These data indicate that the rate of RNA synthesis is relatively high during pre-meiotic interphase and decreases as cells enter meiotic prophase. The total amount of RNA synthesized per cell in pre-pachytene was less than in pachytene. A definite decrease in grain count per cell occurs when cells enter diplotene. Grains are not seen over meiotic divisions nor over the dyad nor tetrad nuclei prior to formation of immature pollen grains.

As the rate of RNA synthesis is decreasing between pachytene and diplotene, a change in the location of the silver grains on the bivalents can be observed. The acrocentric bivalents from cells in late pachytene or very early diplotene are usually labeled from end to end (Figs. 1a, 1b), while bivalents from cells in mid-diplotene (Fig. 2) or late diplotene (Figs. 3a, 3b, 3c) cease synthesizing RNA at the proximal and distal ends.

Table 1. Grain counts from adjacent meiotic stages occupying the same bud

Meiotic stage	Bud no.	$\bar{X}$ total per cell	$\bar{X}$ per nucleolus	Hours in H^3 -uridine	No. cells observed
Premeiotic interphase	A	44.4	17.5	2	30
Pre-pachytene	A	19.3	4.9	2	50
Pre-pachytene	B	19.9	7.3	2	30
Pachytene	B	30.1	6.8	2	30
Pre-pachytene	C	18.8	4.4	2	30
Pachytene	C	35.6	5.8	2	30
Pachytene	D	17.7	2.3	2	30
Diplotene	D	10.6	1.7	2	30
Pachytene	E	44.9	12.4	2	30
Diplotene	E	19.6	4.6	2	30

Figs. 1-3. 1a and 1b, bivalents from cells in late pachytene and early diplotene respectively. Both bivalents are labeled in the proximal, distal and interstitial regions. Both cells were in contact with H^3 -uridine for 2 hours prior to autoradiography. 2, bivalent from cell in mid diplotene. The proximal and distal ends of the bivalent show fewer silver grains than interstitial regions. Cell was in contact with H^3 -uridine for 2 hours. 3a, 3b, and 3c, bivalents from cells in late diplotene. Proximal and distal regions are unlabeled. Silver grains are localized interstitially. Cells were in contact with H^3 -uridine for 2 hours.

Nucleolar RNA synthesis decreases approximately 3 to 4 fold when cells enter prophase of meiosis (Table 1). Cells in pre-pachytene and pachytene appear to synthesize nucleolar RNA at approximately the same rate. The nucleolar RNA synthetic activities appear to decrease during diplotene. However, nucleolar RNA synthesis continues to occur at a low rate until the nucleolus has begun to fragment prior to the first meiotic division (Figs. 4, 5, 6 and 7).

Figs. 4-7. 4, 5 and 6, cells in pre-pachytene, pachytene and the nucleolar organizing bivalent from a cell in diplotene respectively. Arrows indicate labeled nucleoli. Cells were in contact with H^3 -uridine for 2 hours. 7, cell in diakinesis. The three bivalents of *O. virens* ($n=3$) are evident. Note the fragments of the nucleolus which are labeled (arrows). Cells are from a 2 hour sample.

Grain counts cannot be directly compared between the different timed samples due to the variability in uptake by the inflorescences. However, if the counts are transformed to percentage data some information about the pattern of RNA synthesis can be obtained (Table 2). The sample collected at 2 hours shows a different distribution of silver grains in pre-pachytene cells compared to cells in pachytene or diplotene. Pre-pachytene cells show a larger percentage of the total silver grains in the cytoplasm than do cells in pachytene or diplotene where the majority of grains are over the nucleus. The percentage of pachytene cytoplasmic label increases slightly in the 4 and 10 hour samples but at no time reaches the level of pre-pachytene cytoplasmic label.

Actinomycin D

No apparent cytological effects are observed during the first 30 hours of actinomycin D treatment. Samples collected 30 hours after continuous treatment

Figs. 8-13. Cell treated with actinomycin D for 30 hours. Note the fragmented nucleolus (arrows) in this pachytene cell. 9 and 10, control cell and actinomycin D treated cell (30 hours treatment) respectively. Cells are in metaphase II. Note the absence of a cross wall in the actinomycin D treated cell. 11, actinomycin D (36 hours) treated cells in metaphase II. Cells resemble a mitotic metaphase due to the absence of cytokinesis and cross wall formation following anaphase I. 12 and 13, cells in anaphase II ($n=3$) and "tetrad" stage of meiosis respectively. Nuclear division without cytokinesis has occurred due to a 36 hour treatment of actinomycin D.

of actinomycin D show a syndrome of effects. This consists of a generalized swelling of the nucleolus and some nucleolar fragmentation although not all cells show fragmented nucleoli (Fig. 8). A second rather general effect appearing in the treated inflorescences is a loss of meiotic synchrony. In control plants, meiotic

cells are not entirely synchronous within a single bud. There is a degree of synchrony however in that 2 or perhaps 3 adjacent stages may occur together in a single bud. In the treated material, a single bud was frequently seen which contained pre-pachytene cells in addition to later stages such as anaphase I and II.

Table 2. Distribution of labeled ribonucleic acid
(percentage of total silver grains over nucleus and cytoplasm)

Hours with H ³ -uridine	Inflorescence number	Stages					
		pre-pachytene		pachytene		diplotene	
		nucleus* %	cytoplasm %	nucleus %	cytoplasm %	nucleus %	cytoplasm %
2	1	69 ⁺	31	89	11	88	12
2	2	67	33	82	18	95	5
4	3	57	43	79	21	89	11
4	4	70	30	88	12	88	12
10	5	69	31	75	25	86	14
10	6	67	33	82	18	74	26

* Nuclear silver grain counts include both chromosomal and nucleolar silver grains.

+ Each sample represents counts from 30 cells.

A striking effect of actinomycin D is the inhibition of cross wall formation following anaphase I and II (Figs. 9 and 10). Chromosome movement at anaphase I appears normal even after 48 hours of actinomycin D treatment. Thus the spindle apparatus does not appear to be affected by this treatment. Cells enter metaphase II and at anaphase II chromosome movement occurs, again indicating that the spindle is unaltered. However, since no cross wall formation has occurred at anaphase I, metaphase II frequently resembles a mitotic metaphase (Fig. 11) and normal segregation of chromosomes is altered (Fig. 12). The end result can be cells at the "tetrad" stage containing 1, 2, 3 or 4 nuclei (Fig. 11). Under the conditions of these experiments, this effect on cross wall formation always appears 30 to 36 hours after the beginning of treatment.

Discussion

In general a perusal of the literature indicates that relatively high rates of RNA synthesis occurs pre-meiotically (Das *et al.* 1965). Our data are in agreement with this. Many authors have demonstrated that an increase in rate of RNA synthesis occurs in pachytene cells when compared to earlier prophase stages of meiosis or to cells in diplotene (Monesi 1965, Brasiello 1968). Our data are also in agreement with this. Contrary to the above observations, Sauter (1968) and Das (1965) demonstrated decreases in the rate of RNA synthesis during pachytene in *Paeonia* and maize respectively, and Hotta and Stern (1963, 1965) demonstrated a subsequent increase in rate of synthesis during diplotene in *Trillium* and *Tulipa*. In general, all authors agree that fluctuations in the rate of RNA synthesis occur

during different stages of meiosis although slight differences in the pattern of these fluctuations occur depending on the species involved. It should be emphasized that our grain count comparisons were made on adjacent stages occupying the same bud. This was done to eliminate variability in uptake of H^3 -uridine between inflorescences and between buds on the same inflorescence.

Hotta and Stern (1965) attempted to explain the fluctuations in RNA synthesis during meiosis in the tulip. They examined polymerase and nucleoside kinase activities in relation to RNA synthesis and demonstrated that the kinase shows marked variations through the meiotic cycle which parallel those observed for RNA synthesis. This suggested perhaps that nucleoside kinase may be a limiting factor during RNA synthesis.

Heslop-Harrison and Mackenzie (1967) measured the passage of C^{14} -thymidine into the pollen mother cells of *Lilium* during various stages of meiosis and found nearly total exclusion of the nucleoside following pachytene. They suggested that this correlated with the dissolution of the large cytomycytic channels interconnecting the meiocytes and the deposition of a heavy callose layer around each meiocyte. Our results suggest that *Ornithogalum* is not the same as *Lilium* in this respect since the nucleoside H^3 -uridine does readily enter the cells during diplotene. As judged by grain counts, a decrease in RNA synthesis does occur between pachytene and diplotene, but concomitantly a localization of RNA synthesis is noted. Inactivation of definite chromosomal regions (the proximal and distal ends) seems to occur with respect to their ability to participate in RNA synthesis. Thus, less RNA appears to be synthesized during diplotene than during pachytene because fewer chromosomal regions are active, although a change in rate or availability of precursors cannot be ruled out.

Our observations on nucleolar RNA synthesis agree with those of Das (1965) in that we observed a general decrease in nucleolar RNA synthesis during prophase of meiosis. However, RNA synthetic activities of the nucleoli in *O. virens* do continue at a low rate up to the time of nucleolar breakdown. This observation differs from that of Das (1965) who noted complete nucleolar inactivation after pre-pachytene in corn anthers. Lin (1955), using spectrophotometric measurements, noted a doubling of RNA in the nucleoli of maize microsporocytes during leptotene and a small increase during pachytene. Our results are in line with this suggestion. The data that we have accumulated concerning nucleolar RNA synthesis cannot be conveniently compared to data from animal male meiosis. There, the nucleolus is generally very small or absent during mid-prophase which would indicate a major difference in metabolism of the cells. The potentialities of the products, too are quite different—the one yielding microgametophytes and the other sperm.

The distribution of silver grains over the cell (Table 2) shows an interesting pattern. Since the uptake of H^3 -uridine varies between inflorescences (Church unpublished) the data were transformed to percentage data so that comparisons between inflorescences could be made. In the two hour sample, which represents the shortest labeling period, approximately 30% of the label was cytoplasmic in pre-pachytene cells as compared to approximately 15% for pachytene cells and roughly 10% for diplotene cells. The proportion of cytoplasmic label remained

remarkably similar in the 4 hour and 10 hour samples. It should be emphasized, that a 2 hour labeling period is relatively short compared to the length of meiotic prophase in *O. virens* which is approximately 3 days (Church and Wimber 1969). The origin of the cytoplasmic RNA is unknown. It may represent RNA which is synthesized in the cytoplasm or RNA which was transported from the nucleus to the cytoplasm. Whatever the origin of the labeled cytoplasmic RNA, there appears to be a definite difference between the amount present in pre-pachytene cells and cells in pachytene and diplotene. Utakoji (1966) observed that only a small amount of the labeled pachytene nuclear RNA entered the cytoplasm in hamster meiotic cells. However, no observations were made concerning pre-pachytene cells. Muramatsu *et al.* (1968) demonstrated that RNA's synthesized in pachytene nuclei of chinese hamster cells are mostly DNA-like, heterogeneous molecules which apparently are rapidly degraded within the nucleus and thus never enter the cytoplasm. Perhaps much of the rapidly labeled RNA in *Ornithogalum* has a similar fate. However, if the labeled cytoplasmic RNA observed in *O. virens* meiotic prophase cells has its origin in the nucleus, then a greater percentage of the newly synthesized RNA is being transported to the cytoplasm in pre-pachytene cells than in pachytene or diplotene cells.

It is clear that RNA synthesis is occurring during meiosis. It is not clear how this RNA is implicated in meiotic development. Treatment of meiotic cells with actinomycin D has been said to reduce chiasma frequency in *Vicia faba* (Sharma and Singh 1968) and to cause chromosome breakage and suppression of meiosis in a locust (Jain and Singh, 1967). A rather detailed investigation on the effects of actinomycin D in cultured buds of *Trillium erectum* was presented by Kemp (1964). Meiotic delays were observed when cells in early prophase were treated with actinomycin D. We observed a loss of synchrony in meiotic development within a bud; that is, early prophase stages were found to occupy the same bud as meiotic division stages. This observation agrees with those of Kemp. We did not observe an uncoiling of anaphase I chromosomes as reported by Kemp, nor did we observe a malfunctioning spindle apparatus. Treatment of *T. erectum* pollen mother cells with actinomycin D at any time during prophase produced malfunctioning spindle activities (Kemp 1964). The anaphase I cells we observed in the 48 hour sample would have been in zygotene (assuming no meiotic delays) when the actinomycin D treatment was begun. *O. virens* meiotic cells require approximately 48 hours to pass from zygotene to the dyad stage (Church and Wimber 1969). Thus, if RNA synthesized during meiosis is required for proper spindle function in *O. virens*, that RNA must be synthesized prior to zygotene.

Our observations concerning cytokinesis and cross wall formation agree with those of Kemp (1965). Since we first observed upsets in these processes in the 30 to 36 hour samples, we conclude that RNA synthesized during pachytene and diplotene is implicated in cytokinesis and cross wall formation in *O. virens*.

An effect on nucleolar cytology occurred at all stages of meiosis after 30 hours of treatment. This consisted of a generalized swelling of the nucleolus and nucleolar fragmentation. Such effects have been seen in somatic cells treated with actinomycin D for much shorter periods of time (Stevens 1964). Perhaps the long treat-

ment period required to disrupt nucleoli in *O. virens* reflects the low RNA synthetic activity of the nucleolus.

Actinomycin D does not affect synapsis nor chiasma formation. No reduction in chiasma frequency was observed in any of the timed samples. Chiasma frequencies are obtained at diplotene. The cells observed at diplotene in the 48 hour samples were in a prepachytene stage when the treatment was begun. Thus we conclude that RNA synthesized during pre-pachytene, pachytene or diplotene is not required for chiasma formation.

The results of these experiments suggest that RNA synthesis during meiosis is necessary for some aspects of normal meiotic development. Other processes such as synapsis, and chiasma formation are not dependent upon RNA synthesized during prophase of meiosis.

Summary

The pattern of RNA synthesis during meiotic prophase has been investigated in *Ornithogalum virens*. The rate of RNA synthesis fluctuates depending on the stage of meiosis. Nucleolar RNA synthesis continues at a low level throughout meiotic prophase. Evidence is presented which indicates that a greater percentage of the newly synthesized RNA is being transported to the cytoplasm in pre-pachytene stages as compared to cells in pachytene or diplotene of meiosis.

The effects of actinomycin D on the morphology of the meiotic cells are also described.

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