CHANGES IN POLY(A)$^+$ RNA DURING MALE MEIOSIS IN *LILIUM*

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SUMMARY

Levels of poly(A)$^+$ RNA have been investigated at each stage of male meiosis in *Lilium* (var. Firecracker). Two methods were employed in this work: in one extracts from labelled meiocytes were passed through oligo(dT) columns, while in the other the specific probe [^{3}H]poly(U) was hybridized *in situ* with resin-embedded sections of pollen mother cells. The label contained in the eluate from the oligo(dT) columns was measured by liquid scintillation, and the quantity of [^{3}H]-poly(U) hybridized was determined by statistical analysis of light microscopic autoradiographs. Both techniques revealed a dramatic decline in detectable poly(A)$^+$ RNA during prophase. Lowest levels are reached in the pachytene stage, following which a gradual restoration of this species of RNA takes place in both nucleus and cytoplasm. The data presented here provide no clear indication as to whether this fall in RNA levels is caused by the action of novel enzymes specific to the meiotic prophase, by a cessation of synthesis and the activity of normal turnover processes, or by a combination of the two. Although there is some evidence from the [^{3}H]poly(U) hybridization study that a small peak of poly(A)$^+$ RNA synthesis may take place in leptotene, both methods indicate that there is a very low level of poly(A)$^+$ RNA synthesis throughout prophase. The presence of poly(A)$^+$ RNA was not detected in either the accessory nucleoli or the cytoplasmic nucleoloids that characterize the nucleus and cytoplasm of these cells. These events are considered in terms of the juncture at which they occur in the plant life-cycle.

INTRODUCTION

There is accumulating evidence that in angiosperm meiocytes, as the nuclear genome undergoes reduction division, elements of the cytoplasm are also reorganized. The organelle population exhibits a conspicuous cycle of dedifferentiation and redifferentiation (Marumaya, 1968; Dickinson & Heslop-Harrison, 1970*a*; Dickinson, 1981*a*; Bird, Porter & Dickinson, 1982) while, at the same time, quite distinctive changes occur in the metabolism of cytoplasmic RNA. The levels of this RNA drop dramatically in the zygotene–pachytene interval of prophase (Mackenzie, Heslop-Harrison & Dickinson, 1967; Knox, Dickinson & Heslop-Harrison, 1970), accompanied by a partial elimination of ribosomes from the cytoplasm (Mackenzie *et al.* 1967; Dickinson & Heslop-Harrison, 1970*b*). Both ribosome numbers and cytoplasmic RNA levels are restored after the division, and it has been suggested that RNA synthesized in the nucleus during meiotic prophase (Porter, Bird & Dickinson, 1982) may form the basis of new cytoplasmic RNA via the formation and disintegration of cytoplasmic nucleoloids, as in *Lilium* (Dickinson & Heslop-Harrison, 1970*b*), or by

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means of specific nuclear–cytoplasmic interactions, as described for gymnosperms (Dickinson & Potter, 1975).

In the cytoplasm of female meiotic cells, changes closely corresponding to those described for male cells have been noted (Dickinson & Potter, 1978). Such events may be associated universally with meiosis or gametogenesis, or both, and thus represent a mechanism by which information is purged from the sporophytic cytoplasm before the new haploid genome is activated (Dickinson & Heslop-Harrison, 1977; Porter *et al.* 1982). Certainly, the behaviour of organelles at this time indicates some degree of independence from nuclear control (Dickinson, 1981*b*; Bird *et al.* 1982), again suggesting the absence or removal of nuclear-coded informational molecules, presumably messenger RNA.

Eukaryotic mRNA is now well-established as containing a terminal 3′-poly(A) sequence of 50–200 residues (Brawerman, 1974). Using techniques for the identification of mRNA based on hybridization of this poly(A)$^+$ sequence, Appels *et al.* (1982) have investigated the nature of poly(A)$^+$ RNA produced at the pachytene stage of meiosis in *Lilium*. With the aid of cloning techniques these authors have been able to identify a species of poly(A)$^+$ RNA that they regard as specific to meiosis, and report that this RNA is in many ways homologous with RNA formed at meiosis in other species.

We have employed methods based on detecting the poly(A)$^+$ chain of mRNA to examine the levels of poly(A)$^+$ RNA throughout meiotic prophase. Results from this study strongly suggest that RNA removed from male meiotic cells of *Lilium* does indeed include a large proportion of the mRNA pool.

MATERIALS AND METHODS

Plant material

Plants of *Lilium* var. Firecracker (supplied by Gebroeders Van Zanten, Hillegom, Holland) were grown under glass at 20 °C±5 deg. C. As in other species of *Lilium*, the flower buds contain six anthers closely synchronized in their stage of meiotic development until late prophase (Porter *et al.* 1982). To determine the meiotic stage of any bud, one anther was removed and examined after squashing in lacto-propionic orcein. Stages used in these studies ranged from pre-leptotene through to tetrad.

Isolation of ^{32}P-labelled poly(A)$^+$ RNA from Lilium meiocytes

Anthers from various meiotic stages were used as a source of meiocytes for the labelling and isolation of poly(A)$^+$ RNA. Meiocyte filaments were extruded from the anthers, washed and incubated in modified Whites medium (Ito & Stern, 1967) as described previously (Porter *et al.* 1982). Filaments were then incubated for 4 h at 20 °C ± 2 deg. C with 25 µg streptomycin and 100 µCi of [^{32}P]orthophosphate, rinsed three times in fresh medium and the RNA was extracted.

Following rupture in extraction buffer (25 mM-Tris·HCl, pH 7·5, 100 mM-NaCl, 7·5 mM-EDTA, 2·5 % sodium dodecyl sulphate) by passage through a syringe needle (25G) several times, cells were subjected to digestion by proteinase K (Lizardi & Engelberg, 1979). The soluble fraction was extracted several times with phenol/chloroform (3: 1, v/v) containing 5 % isoamyl alcohol and the RNA was precipitated from the aqueous phase at −20 °C by the addition of 2 vol. ethanol. Care was taken to use sterile glassware and solutions where appropriate to minimize ribonuclease contamination. RNA was recovered by centrifugation, washed with 3 M-sodium acetate (pH 6·0) and the poly(A)$^+$ RNA was isolated by binding to oligo(dT)-cellulose (Aviv & Leder, 1972). After

eluting from the oligo(dT) columns, the ^{32}P incorporation into poly(A)$^+$ RNA was estimated by measuring Cerenkov radiation in a liquid scintillation counter.

Electron microscopic autoradiography

Anthers were dissected out of developing buds, cut into 2–3 mm sections and fixed at 4 °C in a mixture of 2·5 % depolymerized paraformaldehyde and 3 % glutaric dialdehyde in 0·05 M-phosphate buffer at pH 7. Material was then dehydrated and embedded in glycol methacrylate and polymerized under ultraviolet light at 1 °C in the absence of oxygen (Spaur & Moriarty, 1977). Sections of the anthers (2 μm) were cut on a Reichert OMU3 Ultramicrotome and dried onto ethanol-washed slides, with five sections mounted close together on each slide. Sections were hybridized with [^3H]poly(U) (Amersham International Ltd) following the method outlined by Raghavan (1981a) using 35 μl of a 2·0 μCi ml^{-1} solution per slide. At the completion of the hybridization procedure, slides were air-dried in preparation for autoradiography.

Several sections from anthers at different meiotic stages were also subjected to pretreatments designed to demonstrate the specificity of binding of poly(U) to poly(A)$^+$ RNA. These include digestion with either DNase (bovine pancreatic, Sigma), RNase A (Sigma, type 1A) or RNase T$_2$ (Sigma, grade VI), and the treatment was carried out as described by Raghavan (1981a) prior to hybridization with [^3H]poly(U).

Autoradiographs were set up to establish the degree of binding of [^3H]poly(U) to the sections. Slides were coated in G4 emulsion (Ilford), which was applied in a monolayer by the loop procedure described previously (Porter *et al.* 1982). After exposure for 8 weeks at 4 °C, the slides were developed (see Fig. 1) and examined under phase in a Leitz Ortholux II microscope.

Analysis of autoradiographs

The autoradiographs were examined for the number and distribution of grains overlying the meiotic cells. Each cell was divided into three components: (a) nucleus, (b) nucleolus and (c) cytoplasm; and the area of each component was estimated using a scaled eye-piece graticule. The number of grains associated with each component was also noted and a background value of grains/100 μm^2 was produced by counting an area of the section where no cells were present. The background value varied by a factor of 5, indicating variation in emulsion thickness between slides.
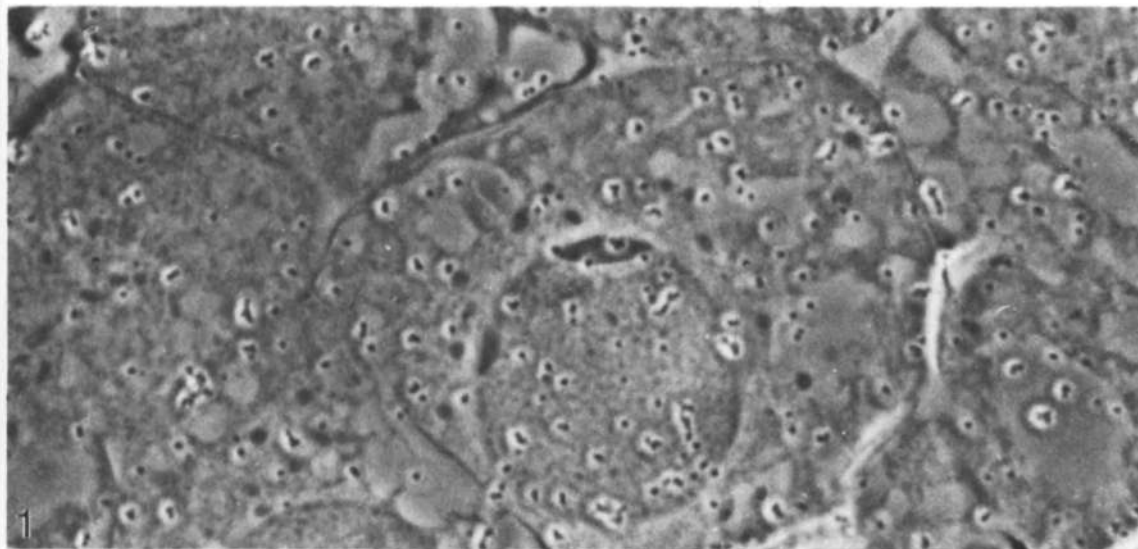

Fig. 1. Autoradiograph showing prophase pollen mother cells of *Lilium* hybridized with [^3H]poly(U), as described in the text. Silver grains are clearly visible. Phase-contrast: approx. ×5000.

The grain count for each cellular component was corrected for the background count estimated for a corresponding area of emulsion on the same slide. The data were then expressed in terms of specific activity:

$$\frac{\text{Number of grains after correction for background}}{\text{Area}}$$

For each meiotic stage the number of cells examined varied from 30–70 until the stages of diplotene and later, when difficulties in fixation left fewer cells available within the sections for analysis. Certain pretreatments also caused sections to peel off the slides during enzymic digestion or the subsequent washing procedures, again resulting in fewer cells in the autoradiographs. Data are thus presented as the mean specific activity together with 95 % confidence intervals, which reflect the sample number.

RESULTS

Isolation of ^{32}P-labelled poly(A)$^+$ RNA

The amount of poly(A)$^+$ RNA labelled with [^{32}P]orthophosphate that can be isolated from these meiotic cells varies considerably as meiosis progresses (see Fig. 2). After leptotene, levels of [^{32}P]poly(A)$^+$ RNA declined, reaching a minimum at the stages of pachytene through to anaphase of the first meiotic division; levels are partially restored as the tetrads develop. The experiment was repeated on three occasions; the specific activity of the isolated poly(A)$^+$ RNA varied each time, but the same changes were seen. Since the period of incorporation of ^{32}P was fairly short only newly
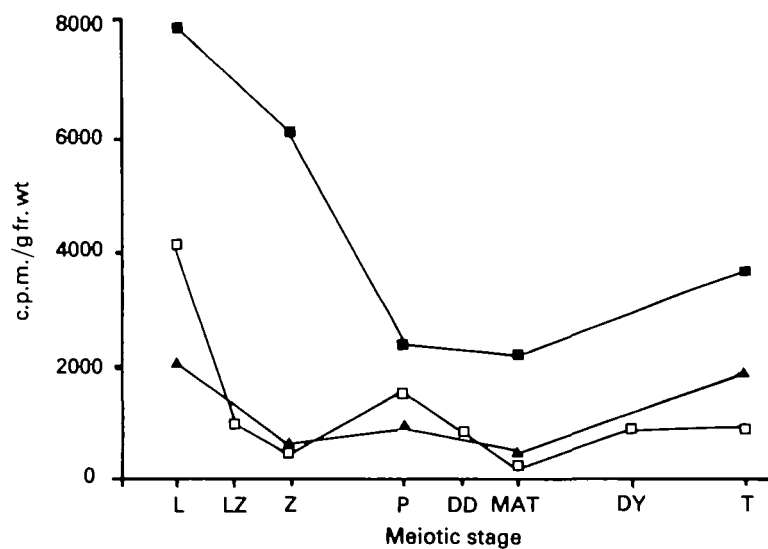

Fig. 2. Specific activity of meiocyte poly(A)$^+$ RNA. Poly(A)$^+$ RNA isolated from *Lilium* meiocytes was labelled with [^{32}P]orthophosphate. The meiotic stages used were: L, leptotene; LZ, leptotene–zygotene; Z, zygotene; P, pachytene; DD, diplotene–diakinesis; MAT, metaphase, anaphase, telophase; DY, dyad; T, tetrad. The three graphs indicate three separate experiments: (▲——▲) exp. 1; (■——■) exp. 2; (□——□) exp. 3. Different meiotic stages were available on each occasion and the data are thus presented separately.

synthesized poly(A)$^+$ RNA will have been labelled, and the changes seen thus suggest that the synthesis of this class of RNA is repressed during certain stages of meiosis.

The isolation of poly(A)$^+$ RNA involved a single step of oligo(dT) purification and it is likely that this fraction of poly(A)$^+$ RNA may contain contaminating ribosomal RNA (Taylor, 1979). In order to establish that poly(A)$^+$ RNA does indeed vary during meiosis, further experiments were carried out using the specific probe, [^{3}H]poly(U).

Hybridization of [^{3}H]poly(U)

The autoradiographs from the hybridization of [^{3}H]poly(U) to sections of *Lilium* anthers were analysed statistically, and the data are presented in Fig. 3. The specific activity of the components gives an indication of the amount of poly(U) bound by that component, taking into account the area it occupies in each of the sections examined. It can be seen that the specific activity of the cytoplasm declines gradually from early prophase to reach a minimum by the stages of pachytene–diplotene and then begins to increase again in the developing tetrads (Fig. 3A). By pachytene it can be seen that the amount of poly(U) bound by a specific area of the cytoplasm has fallen to less than 25 % of the amount bound at the start of prophase.

In the nucleus specific activity shows a similar decline to that of the cytoplasm after an initial, very substantial peak of activity at leptotene (Fig. 3B). There is no noticeable increase in specific activity of the nucleus after pachytene. The nucleolar specific activity was also estimated (Fig. 3C), but since not every section of a meiocyte passes through the nucleus the sample size was somewhat lower than for the other components, and this is reflected in the wider 95 % confidence intervals. Despite the spread of data, there is a significant peak in specific activity at leptotene – in parallel with the nucleus – then a gradual decline until diplotene. No nucleoli were sampled in the sections of dyads and tetrads examined. The grain count over the cells is subject to error arising from 'crossfire', i.e. the distribution of grains in an autoradiograph around a single source of radioactivity. The distribution of grains around a ^{3}H source is, however, very small at the light microscopic level because of the low particle energy; the half distance (the distance from the source within which half the developed grains fall) is between 0·3 and 0·4 μm (Salpeter, Budd & Mattimoe, 1974). Hence it is unlikely to affect results for the cytoplasm or nucleus. In the case of the nucleolus (which can be as small as 5 μm in these sections) there may be some errors from crossfire, but the general trends in poly(U) binding can still be seen.

In the sections where accessory nucleoli or cytoplasmic nucleoloids were observed, there was no significant association of grains with these particular structures.

In order to verify the specificity of poly(U) binding to poly(A)$^+$ RNA, a number of control pretreatments were carried out. The effect of pretreatment with RNase A on the levels of poly(U) hybridization can be seen in Fig. 2. This enzyme, when applied under low-salt conditions, hydrolyses poly(A) segments in RNA (Beers, 1960) and it did cause considerable reduction in poly(U) binding to the cytoplasm (Fig. 3A) and nucleus (Fig. 3B). The data for the nucleolus showed some reduction in specific activity, although the results were variable (Fig. 3C). A similar pretreatment
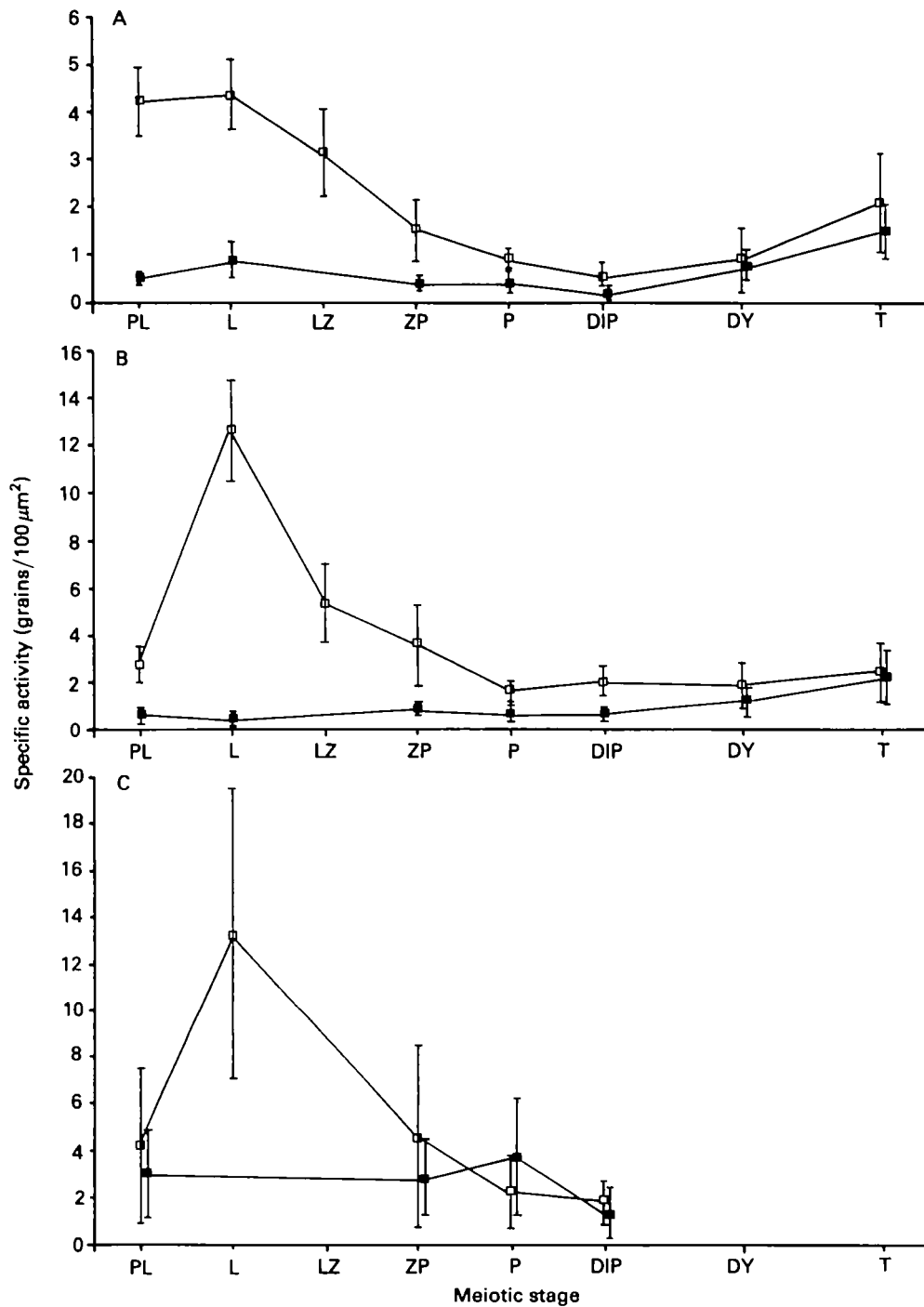
Fig. 3. Hybridization of [³H]poly(U) to sectioned meiotic cells of *Lilium*. A. Cytoplasm; B, nucleus; C, nucleolus. (□——□) Hybridization with no pretreatment; (■——■) hybridization following pretreatment with RNase A. Stages as follows: PL, preleptotene; L, leptotene; LZ, leptotene–zygotene; ZP, zygotene–pachytene; P, pachytene; DIP, diplotene–diakinesis; DY, dyad; T, tetrad.

with ribonuclease T_2, which attacks phosphodiester linkages between polyadenylic residues in RNA (Uclida & Egami, 1971), produced a similar inhibition of binding, and these results are thus not presented here.

Further pretreatments with DNase were also carried out prior to hybridization with poly(U), but the specific activity of cells was only marginally affected and was generally maintained at 75–85 % of the binding to non-pretreated sections. In some cases, pretreatment with DNase even led to an increase in binding of [^{3}H]poly(U) to the nucleus and nucleolus. Similar findings by Raghavan (1981*b*) were attributed to the possible presence of oligo(dA-dT) clusters in nuclear DNA. The negligible effect of DNase in preventing poly(U) hybridization, together with the significant reduction of binding after digestion with RNase, implies that [^{3}H]poly(U) binding to sections does indeed indicate sites of poly(A)$^+$ RNA accumulation within meiotic cells.

DISCUSSION

Changes in poly(A)$^+$ RNA

The decline in, and subsequent restoration of poly(A)$^+$ RNA at zygotene–pachytene revealed by the observations on oligo(dT) binding closely resembles the pattern of removal and resynthesis of total cellular RNA and ribosomes (Mackenzie *et al.* 1967; Knox *et al.* 1970) that also occurs at this time. It seems likely, therefore, that the mechanism that affects total RNA also causes specific falls in the levels of mRNA.

Such an inference is also supported by studies using the specific probe [^{3}H]poly(U) hybridized to poly(A)$^+$ RNA, for analysis of the autoradiographs showed that cytoplasmic levels of mRNA decline gradually as prophase proceeds and start to be replenished as the tetrads develop. In the nucleus poly(A)$^+$ RNA also drops in zygotene–pachytene, although in the leptotene stage a significant accumulation of poly(A)$^+$ RNA was noted, indicating an increased synthesis of this class of RNA at this time. Poly(A)$^+$ RNA in the nucleus is likely to be heterogeneous nuclear RNA, the likely precursor to cytoplasmic mRNA (Dyer & Leaver, 1981). An increased accumulation at leptotene may arise by increased transcription of the genome or increased polyadenylation of previously transcribed RNA. By what means the increase in this leptotene poly(A)$^+$ RNA occurs and its significance are unclear, but it may well constitute a part of the 'meiotic' poly(A)$^+$ RNA recently investigated by Appels, Bouchard & Stern (1982).

By late prophase, levels of nuclear and cytoplasmic poly(A)$^+$ RNA have both declined; hence the reduction in cytoplasmic mRNA may arise from a decreased supply of mRNA precursors from the nucleus. It has previously been surmized that RNA is removed from the cytoplasm by enzymic degradation (Knox *et al.* 1971) and, indeed, as cytoplasmic RNA falls, levels of hydrolytic enzymes in the cytoplasm are known from both cytophotometric (Knox *et al.* 1971) and cytochemical studies (Bird *et al.* 1982) to be high. The present data do not dispute this hypothesis, and indeed such enzymes are most likely to be involved in ribosome elimination. RNA is, however, synthesized in the nucleus at this time, particularly at the nucleolus-organizing

region (Porter *et al.* 1982), and may thus be primarily rRNA, although undoubtedly a low level of poly(A)$^+$ RNA synthesis is maintained. Clearly, the delicate balance between the synthesis and degradation of each species of RNA can be revealed only by detailed turnover studies. Such work is now in progress.

The observation that mRNA can be detected in meiotic cells of *Lilium* contrasts with the findings of Raghavan (1981*b*), who found no significant binding of [^{3}H]poly(U) in microsporocytes of *Hyoscyamus niger*. The tapetal tissue of this plant was, however, very active in binding at certain stages of pollen development and in our sections, also, the tapetal cells were able to bind [^{3}H]poly(U) throughout meiosis. Both *Lilium* and *Hyoscyamus* have secretory types of tapetum and their difference in the binding of poly(U) to meiocytes is difficult to reconcile. It must be recognized, however, that the data for *Hyoscyamus* dealt largely with later stages of pollen development, while the early meiotic stages covered in the present study were hardly considered. The striking decline in total nuclear poly(A)$^+$ RNA reported here is also difficult to reconcile with the synthesis of 'pachytene RNA' reported by Appels *et al.* (1982). Their very elegant work was, however, designed to examine the nature of any poly(A)$^+$ RNA synthesized in pachytene, and there is no doubt that a low level of synthesis does occur. This 'meiotic RNA' may thus constitute a very major proportion of the poly(A)$^+$ RNA synthesized during pachytene.

mRNA and the gametophyte/sporophyte relationship

The changes in poly(A)$^+$ RNA during meiotic prophase support the existence of a general cytoplasmic clearing phase accompanying meiosis (Dickinson & Heslop-Harrison, 1977; Dickinson, 1981*b*). In contrast, it is known that some maternal messages do survive meiotic division and pass into the gametophyte cytoplasm, as is the case of pollen-wall pattern determinants (Godwin, 1968; Heslop-Harrison, 1971; Sheldon & Dickinson, 1983). It has been proposed that some of these messages are protected from any degradation by multi-membraned inclusions that enclose small areas of the pollen mother-cell cytoplasm (Dickinson & Andrews, 1977; Dickinson & Heslop-Harrison, 1977).

Microspores and male gametophytes do, however, show an independent expression of their haploid genome. For example, in *Zea mays*, genes expressed in the pollen include alcohol dehydrogenase (Freeling, 1976), waxy (Nelson, 1962) and several gametophytic (Ga) loci (Bianchi & Lorenzoni, 1975). Pollen tube growth rate has also been shown to be influenced by the gametophyte genotype (Mulcahy, 1979) and recently a microelectrophoretic technique has been developed to allow examination of gametophytic gene products (Mulcahy, Mulcahy & Robinson, 1979). These and other cases of haploid gene expression (Heslop-Harrison, 1979) do suggest that the presence of a mechanism of removing sporophytically coded information from cells that will develop into haploid male gametophytes would be selectively advantageous.

The concept of a re-standardization of the cytoplasm preparatory to the expression of the haploid genome was first developed from genetic studies of fungi by Mather & Jinks (1958). It now would appear that, in higher plants, a very closely regulated 'changeover' from sporophyte to gametophyte takes place in both male and female

cells. Our data indicate strongly that an organized removal of cytoplasmic mRNA takes place during meiotic prophase, but whether this removal occurs solely by the cessation of synthesis and the operation of normal turnover procedures, or by the activities of specific enzymes, remains to be determined. The significance of the fall in ribosome numbers is not easily evaluated, for this appears to vary between plants (Dickinson, unpublished). It may well be that the removal of RNA is only of selective advantage to the plant once that bound to ribosomes is degraded, a process that may involve elimination of a proportion of the ribosome population. In any event, the fresh complement of ribosomes provided by disintegration of the cytoplasmic nucleoloids must surely facilitate early development of the gametophyte.

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