*SYNTHESIS OF DNA DURING MEIOSIS**

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Communicated by Clifford Grobstein, August 17, 1966

Chromosome pairing and crossing-over between homologous chromosomes in meiotic cells are two distinctive events which must in some way involve the DNA moiety of the chromosome. Ever since the unambiguous demonstration by Taylor and McMaster[1] that DNA synthesis occurs during premeiotic interphase, explanations of pairing and crossing-over in terms of DNA synthesis during meiotic prophase have been more or less laid aside. At least one case of thymidine incorporation during meiotic prophase, has been reported.[2] However, this report has been treated casually, either because of the very few silver grains in the radioautographs or because of the possibility that the synthesis was no more than the extension of an otherwise conventional S-period. ("S-period" refers to period of DNA synthesis.)

This communication demonstrates the actuality of DNA synthesis during meiotic prophase and its separation in time from the premeiotic S-period. The materials used in these studies were the microsporocytes of liliaceous plants. The advantages which such materials offer for a correlated study of biochemical and cytological events have been discussed in several earlier publications.[3]

Methods.—Meiotic cells were labeled either by exposing terminal segments of stems to appropriate solutions as described by Taylor and McMaster[1] or by explanting meiotic cells into suitable culture media. White's basic medium (salts and vitamins) supplemented with 0.3 *M* sucrose was used for lily microsporocytes, and a medium of similar composition made solid with agar was used for trillium. Details of culture techniques will be published separately. Bacterial contamination, although always a serious hazard in cell cultures, was not a factor in these experiments. Although various tests were run to check for contamination, the most convincing proof that the labeling was in microsporocyte DNA is to be found in the specificity of hybridization (Fig. 6). Cytological stages were determined by direct microscopic observation or, in some cases, by determination of bud length.[4] The nuclei were isolated and DNA was purified by the methods of Hotta *et al.*[5] The most demanding part of the procedure was the removal of adhering tapetal nuclei from microsporocytes prior to fractionation. In the case of bud cultures, incomplete removal seriously affected results because tapetal cells synthesize DNA during the interval when microsporocytes are in the prophase of meiosis. This problem was solved by extruding the microsporocytes into a solution of trypsin (50 μg/ml) for 5 min at room temperature. This treatment destroyed tapetal nuclei and intact microsporocytes were then recovered by centrifugation. In the case of cultured microsporocytes, contamination by tapetal nuclei was a minor problem. Not only are the nuclei dislodged from the coherent mass of microsporocytes in liquid culture, but they do not incorporate label. Thus, for cultured cells, trypsin treatment proved to be superfluous.

Density analyses of the isolated DNA were carried out according to the procedure of Messelson *et al.*[6] Preparative runs with solutions of cesium chloride were made in siliconized 5-ml tubes and centrifuged at 25°C for 3–4 days. At the termination of each run, samples were collected by puncturing the tubes. DNA was hydrolyzed into its component nucleotides by digestion with DNase and phosphodiesterase.[7] The deoxynucleotides were resolved by paper electrophoresis in 0.05 *M* ammonium acetate buffer (pH 3.7). DNA-DNA hybridization studies were carried out by fragmenting the DNA according to the procedure of McCarthy and Bolton and by adsorbing one of the DNA preparations to a membrane filter as described by Gillespie and Spiegelman.[8] DNA-bound membrane filters were placed in scintillation bottles containing labeled DNA in 5 ml of 0.9 *M* NaCl:0.015 *M* citrate (6 SSC) and incubated at 66°C. After the annealing period, membrane filters were washed with 100 ml of 6 SSC, treated with RNase (20 μg/ml), and again washed with 6 SSC.

Results.—When intact flower buds of *Lilium longiflorum* or of *Trillium erectum* were exposed to P^{32}-phosphate for 24 hr during different intervals of the meiotic cycle, the following results were obtained: DNA isolated from cells in the premeiotic S-period was heavily labeled and that isolated from cells in zygonema and pachynema was lightly labeled. No appreciable amount of label was found in the DNA of cells exposed to isotope either between the termination of the S-phase and late leptonema or at any meiotic stage after pachynema. Identical results were obtained with four varieties of *L. longiflorum*. No exceptions have yet been found to this pattern of behavior. A typical density analysis of DNA which had been labeled during meiotic prophase is shown in Figure 1. The evidence that the radioactivity is DNA may be summarized thus: (1) the pattern was abolished by treatment with DNase, but unaffected by a protease or RNase; (2) labeled deoxynucleotides were isolated by hydrolysis; (3) labeling with tritiated thymidine yielded similar patterns. From these results, we inferred that DNA synthesis during the zygotene-pachytene stages of meiosis was real and that it occurred as a discrete event.

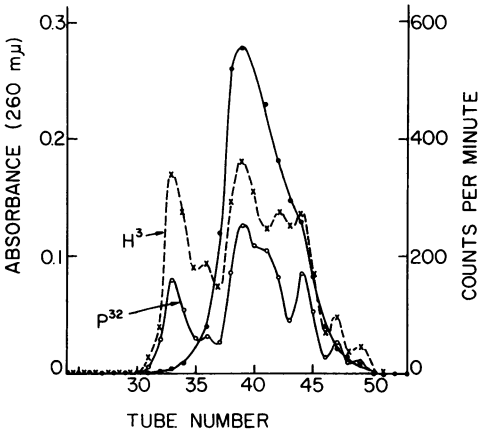

FIG. 1.—Density distribution of DNA isolated from microsporocytes of *Lilium longiflorum* (var. Nellie White). Intact buds containing zygotene cells were cultured for 24 hr in the presence of P^{32}-phosphate (20 μc/ml). To obviate the problem of contamination by tapetal nuclei, the microsporocytes were extruded and cultured *in vitro* for an additional 3 days in cold phosphate medium. Experiments in which DNA was prepared from microsporocytes immediately after extrusion invariably introduced some tapetal DNA in the product, but the results obtained were essentially the same as those illustrated in the figure. The procedure for removing tapetal DNA is discussed under *Methods*. The P^{32}-pattern should be compared with the H^3-thymidine curve which was obtained by extruding microsporocytes at zygonema and culturing these for 4 days at 20°C. Several peaks of radioactivity may be noted and their correspondence in density under the two different conditions of labeling should be observed. The unmarked curve tracks the absorbance values and thus represents the density distribution of the total DNA.

Persistence and distinctiveness of labeled DNA: Further experiments were undertaken with explanted microsporocytes which have the advantage of more efficient isotope utilization and of reduced interference by tapetal cells (see *Methods*). Lily microsporocytes exposed to P^{32}-phosphate *in situ* or to H^3-thymidine *in vitro* showed similar labeling patterns in the DNA component (Fig. 1). The same correspondence in labeling patterns between meiotic cells maintained in the bud and those maintained in culture was found for *Trillium erectum*. These results, combined with the evidence that meiotic cells explanted after pachynema did not incorporate label into their DNA, adequately demonstrated that the normal cytological development of cultured meiotic cells was matched by a normal course of DNA synthesis.

In order to determine whether the prophase labeling was transient or persistent, intact flower buds were exposed to radioactive phosphate for 24 hr. The microsporocytes were then explanted and cultured for 3 days in a medium containing cold

phosphate. No significant difference in the degree of DNA labeling was found between cells so treated and those harvested immediately after labeling (Fig. 1, legend). A similar comparison using cultured cells gave the same results. The method would not reveal a turnover in the DNA which was less than 20 per cent of the initial label, but within this limit the conclusion may be drawn that the DNA labeled during meiotic prophase persists at least until the termination of meiosis. A more detailed characterization of the labeling process was undertaken to determine whether the distinctive pattern of density distribution was constant throughout the labeling period or whether it altered with the progress of the meiotic cycle. Lily microsporocytes were explanted at four different stages of meiotic prophase and harvested after being exposed to isotope for 20 hr (Fig. 2). The results show that the labeling pattern varies with the progress of meiosis. The variations, however, are not random. The positions of the labeling peaks in the density gradient are constant for all stages; the differences between stages relate to the height of the peaks. The results thus show a cycle in the intensity of DNA labeling during meiotic prophase which reaches a maximum in late zygonema or early pachynema (Fig. 2*B*). This cycle appears to be a composite of overlapping individual cycles which characterize each of the peak positions. The significance of the low level of labeling after the termination of pachynema (Fig. 2*D*) is unclear since it could be attributed

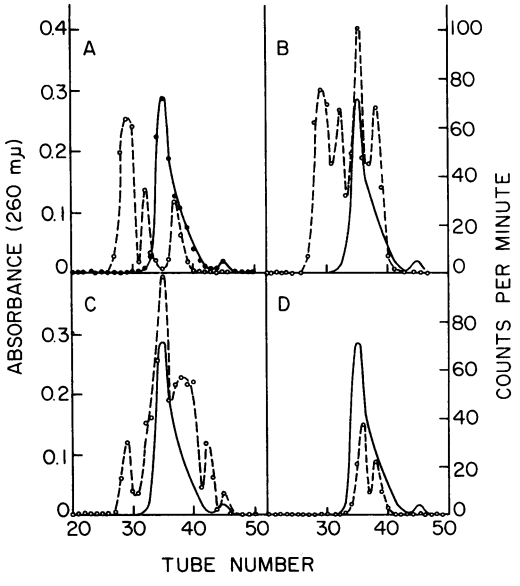

FIG. 2.—Sequence of DNA labeling patterns during the prophase of meiosis in lily (Nellie White). Microsporocytes were cultured for 24 hr before P^{32}-phosphate was added to the medium (10 μc/ml). The cells were harvested after 20 hr of development in the presence of isotope. The stages during which the cells were exposed to isotope are as follows: (*A*) zygonema; (*B*) late zygonema, early pachynema; (*C*) late pachynema; (*D*) late pachynema, early diplonema. Each DNA preparation was run separately in a cesium chloride solution. All curves have been normalized with respect to *A* for specific activity and density of the DNA. Since, in the case of Fig. 1, the microsporocytes were exposed to label during most of the zygonema-pachynema period, the curve shown in that figure may be regarded as a composite of the curves shown here. Radioactivity is shown by broken lines and UV absorbancy by solid lines.

to the small degree of asynchrony in the cell population. The results further emphasize the distinctiveness of DNA synthesis during meiotic prophase.

In order to test more thoroughly for the selective labeling of DNA during meiotic prophase, we examined cells of *Trillium erectum* in which zygonema is separated from the premeiotic S-phase by several months rather than 3–4 days. A comparison between the labeling patterns of the DNA in trillium microsporocytes explanted at premeiotic interphase and at zygonema-pachynema, respectively, is shown in Figure 3. As would be expected, labeling during the S-phase tracks the optical density pattern. On the other hand, almost all of the DNA labeled during prophase is on the heavy side of the gradient. The differences in labeling patterns between lily and trillium may be fortuitous, but both patterns contribute to the conclusion that meiotic prophase is characterized by a distinctive type of DNA synthesis.

Amount of DNA synthesis: The amount of radioactivity incorporated into the DNA during meiotic prophase is small compared with that incorporated during the premeiotic S-phase (Fig. 3). An attempt was made to determine the total amount of DNA synthesized by exposing microsporocytes to either H^3-thymidine or C^{14}-bromodeoxyuridine of known specific activities. Since the base composition of the newly synthesized DNA was known (see below), the amount of DNA synthesized was calculated to be 0.348 per cent based on thymidine incorporation and 0.302 per cent based on BUDR incorporation. Although the value of 0.3 per cent is approximate, the possible margin of error cannot be great enough to alter the conclusion that the DNA synthesized during meiosis represents a very small fraction of the total DNA. Attempts to demonstrate the synthesis by autoradiographic techniques led to equivocal results. No localized labeling was found, but

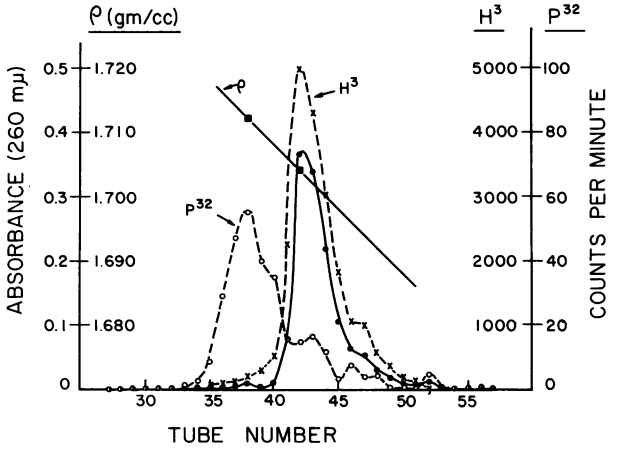

FIG. 3.—Density distribution of labeled DNA obtained from microsporocytes of *Trillium erectum* which had been exposed to H^3-thymidine during the premeiotic S-phase or to P^{32}-phosphate during zygonema-pachynema. Cells cultured at meiotic prophase were allowed to complete meiosis before harvesting. The premeiotic cells reverted to a mitotic division. This phenomenon of reversion to mitosis and its control under the stimulus of explantation will be reported separately. The important feature of the figure in relation to this communication is the distinct difference in patterns between labeling of DNA during the S-phase and during the prophase of meiosis. UV absorbancy is represented by the solid curve. The density values (gm/cc) of each of the samples is indicated by the straight line.

TABLE 1

SPECIFIC ACTIVITIES OF DNA LABELED DURING MEIOTIC PROPHASE AND PREPARED FROM EITHER ISOLATED NUCLEI OR UNFRACTIONATED CELLS

Stage of labeling	Two Days		Four Days	
	Whole cells	Nuclei	Whole cells	Nuclei
Zygonema	58	60	94	92
Pachynema	26	21	18	22

Numbers represent cpm/μg DNA. The meiotic cells were exposed for either 2 or 4 days to P^{32}-phosphate.

the number of grains observed over the nuclei which were susceptible to DNase were only slightly higher than background and could not be regarded as an independent proof of DNA synthesis.

Localization of DNA synthesis: Since the possibility existed that the labeled DNA was cytoplasmic, tests were made on isolated nuclei from microsporocytes which had been exposed to label from zygonema to the termination of meiosis. Two types of analyses were made on the DNA isolated from these nuclear fractions: banding in a cesium chloride gradient and determinations of specific activities. The banding pattern obtained was virtually identical with that of the labeled DNA extracted from whole cells. The data on specific activities are shown in Table 1. Clearly, the specific activity of the DNA prepared from purified nuclei was similar to that prepared from whole cells. We consider these results to be an adequate demonstration of nuclear rather than cytoplasmic labeling.

Physicochemical nature of the product: Samples of DNA were denatured either by treatment with alkali or by heat, and were then banded on a cesium chloride gradient. Both the optical density and the radioactivity peaks of the denatured samples shifted to heavier densities (Fig. 4). We considered these results to indicate

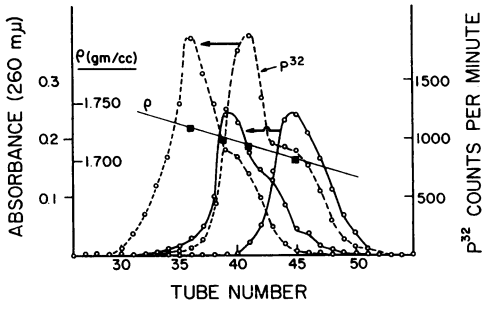

FIG. 4.—Effect of denaturation on density distribution of DNA from microsporocytes *L. longiflorum* (Nellie White). Microsporocytes were exposed to label during meiotic prophase. In one sample the DNA was denatured by treatment with alkali and then centrifuged in a cesium chloride solution. The other sample was similarly centrifuged without prior treatment. The two curves have been superimposed on the basis of the refractive indices of individual fractions. The density shifts due to denaturation are indicated by the arrows. Labeled and bulk material, represented by broken and solid lines, respectively, show the same behavior, thus indicating that the DNA synthesized during the prophase of meiosis is a double-stranded product.

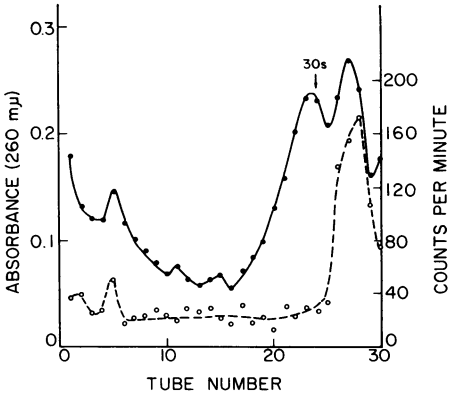

FIG. 5.—Sedimentation properties of prophase-labeled and bulk DNA. The purified DNA was layered over a 5–25% sucrose gradient prepared on a 1 *M* sucrose cushion (5 ml). Centrifugation was carried out at 2°C for 4 hr at 25,000 rpm in a Spinco SW-25 rotor. Labeled 30*S* ribosome particles prepared from *E. coli* were included to serve as a marker. The position of the ribosome peak is indicated by the arrow. Solid line represents absorbancy values (total DNA); dotted line represents radioactivity.

TABLE 2

BEHAVIOR OF NATIVE AND DENATURED DNA PREPARED FROM LABELED MEIOTIC CELLS ON MEMBRANE FILTERS

	Native		Heat-denatured	
	Absorbance	Cpm	Absorbance	Cpm
Trapped	—	0.5	0.520	612
Passed	0.420	518	0.166	71

Used in these experiments were 16.7 μg of native DNA with a total of 568 cpm and 20.8 μg of heat-denatured DNA with a total of 710 cpm. DNA was labeled by exposing meiotic cells in prophase to P^{32}-phosphate.

that the newly synthesized DNA, like the bulk of the DNA, was double-stranded. The conclusion was confirmed by passing untreated and heat-denatured DNA samples through nitrocellulose membrane filters (Table 2). Since the prophase-labeled DNA was double-stranded, its higher density would be expected to be matched by a higher GC content. The expectation was confirmed when enzymatic digests of DNA were resolved by paper electrophoresis (Table 3). The deoxynucleotides labeled during meiotic prophase had a higher GC content than those labeled during premeiosis or those obtained from the bulk of the DNA.

When DNA from prophase-labeled cells was analyzed for its sedimentation properties on a sucrose gradient, most of the newly synthesized DNA had a lower sedimentation value than the total DNA (Fig. 5). A small but variable fraction of the radioactive DNA showed a relatively high sedimentation value. Three cultures were thus analyzed and all showed similar patterns. Chemical analysis of different portions of the gradient with a view to finding some correlation between molecular weight and base composition led to indifferent results. The compositions were all similar to those shown in Table 3. The only conclusion we could draw from these studies was that under the conditions of isolation employed, DNA synthesized during meiotic prophase had a lower average molecular weight than that of total DNA. Whether the observation reflects a real difference in molecular weights or a difference in lability of certain internucleotide linkages is unknown, but the results do point to some physical distinctiveness of the DNA synthesized at that time.

Relationship of DNA formed during meiosis to somatic DNA: In order to determine whether the DNA formed during meiosis was peculiar to meiotic cells or whether it was a normal component of all cells, a series of hybridization tests was carried out. DNA prepared from isolated somatic nuclei of lily was adsorbed to membrane filters as described under *Methods*. The respective capacities of DNA labeled during the S-phase (i.e., totally labeled) and DNA labeled during meiotic prophase to hybridize with somatic nuclear DNA were compared in three ways (Fig. 6). The time course of hybridization was followed (Fig. 6*A*); in both cases

TABLE 3

BASE COMPOSITION OF TOTAL DNA AND OF DNA LABELED DURING MEIOTIC PROPHASE

	Deoxycytidylate	Deoxyguanylate	Thymidylate	Deoxyadenylate
Total DNA (M %)	20.2	21.4	29.6	28.9
Premeiotic DNA (% radioactivity)	20	22.8	30.2	27.0
Meiotic DNA (% radioactivity)	24.8	25.0	26.2	24.0

Values for total DNA were obtained from the radioactive preparations but were based upon absorbancy measurements of the individual 5′-deoxynucleotides. Radioactive preparations were obtained by exposing either premeiotic cells during S-phase or meiotic cells during prophase to P^{32}-phosphate.

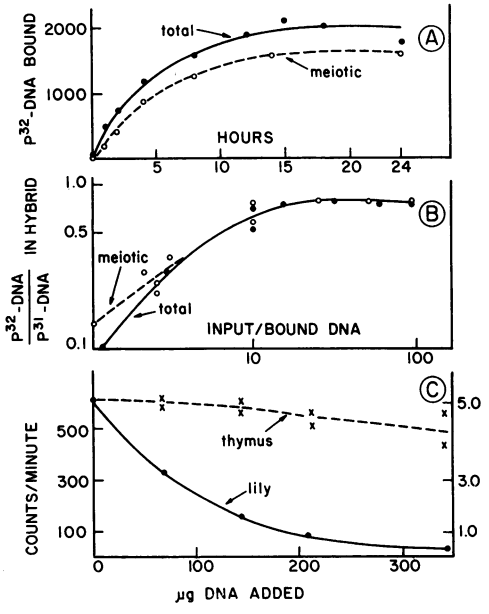

FIG. 6.—A comparison of hybridization behavior between DNA isolated from somatic cells and the DNA synthesized during meiotic prophase. Unlabeled DNA derived from somatic nuclei was adsorbed to membrane filters as described in the text. Labeled DNA was prepared from somatic and meiotic cells of *Lilium longiflorum*. (*A*) Time course of hybridization reaction. (*B*) Ratio of labeled/unlabeled DNA in the hybrid in relation to the concentration of labeled input DNA. The virtual identity in patterns between labeled DNA derived from somatic cells (*total*) and that derived from cells labeled during meiotic prophase should be noted. (*C*) Competitive effect of unlabeled somatic DNA from lily and from calf thymus on hybridization of DNA labeled during meiotic prophase.

the reaction was completed within approximately 15 hr. The ratio of the two DNA components in the hybrid at saturation levels was determined (Fig. 6*B*); in both cases, approximately 1:1 ratios were obtained. The competitive effect of somatic DNA on the hybridization reaction of prophase-labeled DNA was examined (Fig. 6*C*). Somatic DNA of lily was clearly competitive; that of calf thymus was not. From these analyses, we concluded that the DNA synthesized during meiotic prophase is present in somatic nuclei.

Functional significance of DNA synthesis during meiotic prophase: In order to demonstrate that DNA synthesis during meiosis was essential to the development of the meiotic cell, a series of studies was carried out with inhibitors of DNA synthesis. These will be fully described elsewhere, but two of the principal results may be mentioned here in order to clarify the significance of these studies. When deoxyadenosine was added to zygotene cells so as to inhibit DNA synthesis, meiotic development was totally arrested. When deoxyadenosine was added after the initiation of synthesis, the chromosomes became extensively damaged. We regard these cytological effects as adequate proof of the proposition that DNA synthesis during zygonema-pachynema is an essential component of the meiotic process.

Discussion.—The fact of DNA synthesis during meiotic prophase is reasonably clear. Not only does it occur as a discrete event but the product formed has distinctive physicochemical properties. The timing of the event is such that it overlaps with the interval of chromosome pairing and extends beyond it. The simplest interpretation of this observation is that crossing-over occurs during meiotic prophase and that DNA synthesis is a consequence of break-repair mechanisms. Without excluding this simple interpretation, the possibility may be raised that at least part of the DNA synthesized during meiotic prophase is a chromosomal component which was not replicated during the premeiotic S-period. All the evidence obtained is consistent with this interpretation, and two arguments might

be advanced which would favor it: (1) If crossing-over is a nearly random process, we would not expect a pattern of labeling which differed in composition from the total DNA. Only if crossing-over were limited to distinctive regions of the chromosome might the observed distribution of label be expected. Such a limitation is difficult to reconcile with the simple model of a chromosome as an extended DNA filament. (2) To explain the arrest of meiosis by DNA inhibitors applied at zygonema-pachynema on the basis of an inhibition of the repair mechanism seems farfetched. A more logical explanation is that completion of DNA synthesis is essential to meiotic development.

On the basis of these considerations and other observations to be reported later, we favor the interpretation that the DNA synthesized during the prophase of meiosis represents a delayed replication of part of the chromosome. This implies that in the case of mitosis such replication is not delayed and that it perhaps occurs in the late "S" or early G-2 phase. The interpretation thus proposes that one major determinant in the differentiation of a meiotic from a mitotic cell lies in the regulatory mechanism which delays the reproduction of some essential DNA component of the chromosome. We speculate that this component is *functionally* an axial element and that such crossing-over as does occur during meiosis may be closely associated with the delayed reproduction of this element.

* This work was supported by a grant from the National Science Foundation (NSF-GB-3902).

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