SYNTHETIC CAPACITIES OF CHROMOSOME FRAGMENTS CORRELATED WITH THEIR ABILITY TO MAINTAIN NUCLEOLAR MATERIAL

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ABSTRACT

Onion (*Allium cepa*) and bean (*Vicia faba*) root tip cells containing many micronuclei, derived from x-ray–induced chromosome fragments, were exposed to H^3-thymidine and H^3-cytidine to determine the ability of such fragments to undergo DNA and RNA synthesis. Only a few micronuclei in onion and many in bean roots synthesize nucleic acid simultaneously with their main nuclei. A few micronuclei labeled with H^3-thymidine undergo mitotic chromosome condensation along with the main nuclei, while the unlabeled ones never do so. The onset of nucleic acid synthesis as well as mitosis in micronuclei appears to be under generalized cellular control. Although all chromosomes and chromosome fragments at telophase give a positive reaction for a silver stainable nucleolar fraction, in the subsequent interphase only some micronuclei, derived from such chromosome fragments, are found to maintain nucleoli; others lose them with time. Those micronuclei which maintain nucleoli, perhaps due to the presence of specific chromosomal regions, are also active in DNA and RNA synthesis. These results are compatible with the concept that nucleoli and associated chromosome regions play an important role in the primary biosynthetic processes of the cell.

It has been observed by La Cour (1953) and McLeish (1954) that the ability of micronuclei, derived from chromosome fragments, to enter mitosis depends on the presence of nucleolar organizers in them. Similarly, Gaulden and Perry (1958) reported that the inactivation of nucleoli of grasshopper neuroblast cells by microbeam ultraviolet rays leads to mitotic inhibition. Such studies coupled with those on the behavior of nucleoli during mitosis (see reviews by Montgomery, 1898; Gates, 1942; Swift, 1959) suggest that the nucleolus, with its associated chromosome regions, plays a significant role in the events that lead to cell division (Mazia, 1961). That the nucleolus is involved in RNA and protein metabolisms has long been postulated and demonstrated by some recent studies (see reviews by Vincent, 1955; Brachet, 1957; Swift, 1959; Sirlin, 1960; Mazia, 1961).

We have previously reported (Das and Alfert, 1959; Das, 1962) that all chromosome fragments induced by x-rays in onion and bean root meristems give a positive reaction for silver-stainable nucleolar granules at telophase; all micronuclei at early interphase contain some of this reactive material. The present study, which has been undertaken to determine the ability of chromosome fragments to synthesize DNA and RNA after they form micronuclei in the cytoplasm, reveals that only those micronuclei which are capable of maintaining nucleolar bodies are active in synthesis of nucleic acids.

MATERIALS AND METHODS

Onion (*Allium cepa*) and bean (*Vicia faba*) root meristems were x-rayed for 4 minutes at a dose rate of 50 r per minute (at 250 kv and 15 mA, using 0.25 mm Cu and 1.0 mm Al filters; see Das and Alfert, 1961) to

induce chromosome fragmentation. After complete mitotic recovery, which occurred in these materials about 16 hours postirradiation, many micronuclei were formed from such chromosome fragments. Most of the micronucleate cells that originate at the time of mitotic recovery are likely to be in DNA synthesis 7 to 12 hours later, since the pre-DNA synthetic period (G_1) takes about 3 to 7 hours in these materials (Howard and Dewey, 1960; Das and Alfert, unpublished data). Twenty-six hours following irradiation with 200 r, roots were exposed to tritiated thymidine (0.5 and 2.0 μc/ml; sp. act. 3 c/mM) for 2 to 24 hours to study DNA synthesis in the main nuclei and micronuclei. RNA synthesis in these nuclei was also studied by exposing another batch of similar roots to tritiated cytidine (10 μc/ml; sp. act. 1.9 c/mM) for 1 hour.

All samples were fixed in acetic-alcohol and the incorporation of H^3-thymidine and H^3-cytidine into DNA and RNA, respectively, was determined by stripping film autoradiography (Doniach and Pelc, 1950). Prior to application of film, roots exposed to H^3-thymidine were hydrolyzed in 1 N HCl at 60°C for 5 minutes, squashed, and stained with Feulgen, while roots containing H^3-cytidine were squashed following pectinase digestion (Woodard *et al.*, 1961). These cell preparations were then stained, through the processed film, with 2 per cent aqueous solution of methyl green, or, when DNA was removed, with a 1:4 mixture of 2 per cent pyronin and 0.1 per cent alkaline Fast green. After appropriate nuclease digestion it was observed that H^3-thymidine was incorporated into DNA, while the incorporation of H^3-cytidine in micronuclei was predominantly into RNA under the present experimental conditions. To ensure detection of labeled micronuclei, the films were exposed long enough to produce a heavy overall grain pattern over the main nuclei. A dual film exposure time used after H^3-thymidine incorporation into bean roots (see legend to Table I) indicates that the frequency of DNA-synthesizing micronuclei is adequately determined by these procedures. Such tests of film exposure for an underestimation of H^3-cytidine incorporation into micronuclei were not possible because of the interference by cytoplasmic radioactivity (also see Results(*b*)).

In order to compare the sizes of labeled and unlabeled micronuclei, their diameters were measured on camera lucida drawings. The presence or absence of nucleolar bodies in labeled and unlabeled micronuclei was determined on the same slides, after scoring the autoradiographs, by the sensitive technique of silver staining described previously (Das, 1962). By this technique it is possible to detect in various plant and animal cells even small amounts of a non-RNA nucleolar fraction, which appears to be distributed into the cytoplasm at prophase and which eventually seems to reaggregate around chromosomes at telophase, finally forming interphase nucleoli. Since methyl green or pyronin–Fast green stain was lost during the silver staining procedure, it was necessary to restain the cells following silver staining of nucleoli.

All the autoradiographs in Figs. 1, 2 *a*, 3 and 4, of Feulgen-stained squash preparations, were exposed for 2 weeks.

FIGURES 1 AND 2 *a*

Autoradiographs of onion (Fig. 1) and bean (Fig. 2 *a*) root tip cells showing unlabeled and labeled main nuclei and micronuclei (*m*) after 2 hours' exposure to H^3-thymidine (2.0 μc/ml). × 1200.

FIGURE 2 *b*

Silver staining of the same bean cells presented in Fig. 2 *a*; note that the nucleolar material is present only in labeled micronuclei. × 1200.

FIGURES 3 AND 4

Autoradiographs of mitotic micronucleate cells from bean roots, exposed to H^3-thymidine (2.0 μc/ml) for 4 hours and fixed 6 hours after further growth in unlabeled thymidine; Fig. 3 shows that the labeled micronucleus (*m*) is in mitosis along with the main nucleus; Fig. 4 shows that the unlabeled and labeled micronuclei (*m*) are in interphase, and the main nucleus is in mitosis. Fig. 3, × 1500; Fig. 4, × 1750.

FIGURE 5

Bean cells, showing that larger micronuclei (*m*) are diffusely and lightly stained with Feulgen. × 960.

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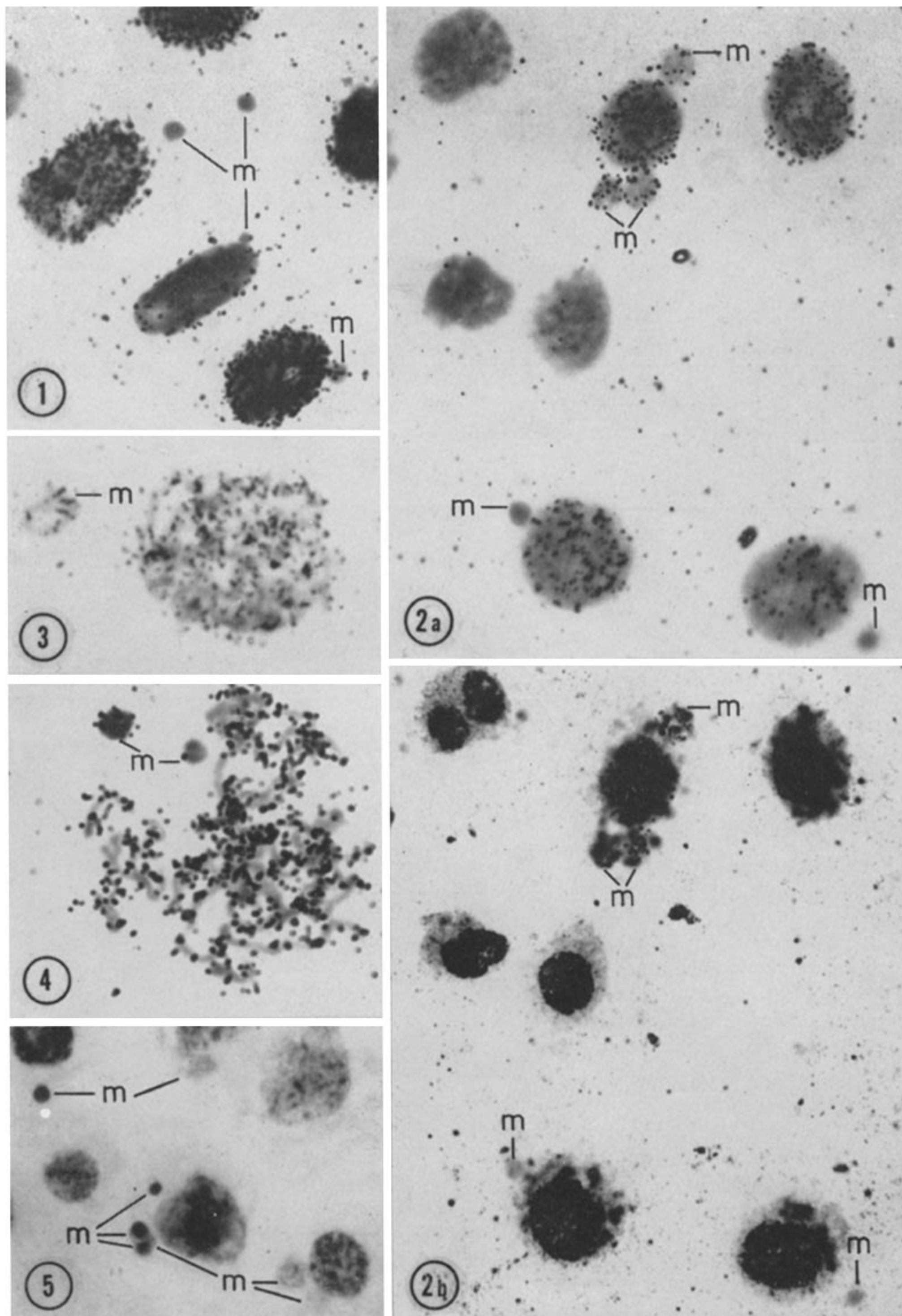

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RESULTS

The following observations are based on cells containing micronuclei; henceforth, these will be referred to as MN cells.

(a) *DNA Synthesis:*

Up to about 33 per cent of interphase MN cells in onion are found to be labeled in the main nuclei following an exposure to H^3-thymidine for 2 to 4 hours (Table I). Among these labeled cells only 1 to 2 per cent are also labeled in micronuclei (Table I; Fig. 1). No MN cells are found to incorporate H^3-thymidine exclusively into micronuclei. The frequency of labeled MN cells of onion roots (Table I, column 4) does not increase between 4 and 9 hours' exposure to H^3-thymidine, probably because of the continued formation of new MN cells during this period. On the other hand, among all labeled MN cells, the frequency of those labeled in both main nuclei and micronuclei increases in roots exposed to H^3-thymidine for 9 hours, at which time many labeled telophases are seen (Table I). Some of this increase, therefore, may be due to the formation of labeled micronuclei from intranuclear chromosome fragments that synthesized DNA prior to their expulsion into the cytoplasm during mitosis. When the exposure of roots to H^3-thymidine is continued for a total of 9 to 24 hours and labeled mitotic cells are scored, it is found that 52 cells, out of 100 with mitotic abnormalities, contain labeled fragments. This suggests that chromosome fragments which have remained in the nucleus since irradiation are more likely to undergo DNA synthesis than those that have been excluded from the nucleus in the course of a previous mitosis (*cf.* also McLeish, 1954).

In contrast to onion, bean roots exposed to H^3-thymidine for 2 to 4 hours (26 hours after x-irradiation with 200 r) contain a greater proportion of labeled MN cells, as well as cells labeled in both main nuclei and micronuclei (Table I). Similar to onion micronuclei, bean micronuclei are found to be labeled only in cells in which the main nuclei are in the process of DNA synthesis. MN cells containing more than one labeled micronucleus are frequently encountered in this material (Fig. 2 *a*). Only a few of the labeled micronuclei undergo mitotic chromosome condensation along with main nuclei, while the unlabeled ones never do so (Figs. 3 and 4). In roots exposed to H^3-thymidine for 4 hours and then grown in tap water containing unlabeled thymidine (0.1 mg/ml) for 6 to 10 hours prior

TABLE I

DNA Synthesis in Micronucleate Interphase Cells

Exposure time to H^3-thymidine (26 hrs. after x-irradiation with 200 r)		Micronucleate cells:			% of (+) micronucleate cells labeled in:		No. (+) telophases out of 100 scored
		Total	No. (+)	% (+)	Main nucleus	Both main nuclei and micronuclei	
	(*hrs.*)						
Onion	2	260	63	24.2	98.4	1.6	—
	4	280	91	32.5	98.9	1.1	0
	9	283	90	31.8	94.4	5.6	36
Bean	2	1090	658	60.4	66.4	33.6	0
	4	1031	680	66.0	59.3	40.7	8

Cells were scored at each time from 2 onion and 4 bean roots which were grown in 0.5 and 2.0 μc/ml of H^3-thymidine, respectively; autoradiographs of Feulgen-stained squash preparations of onion and bean roots were exposed for 4 and 2 weeks, respectively. One sample of bean root cells grown in H^3-thymidine for 2 and 4 hours was also exposed for 4 weeks to produce a very high grain density over the main nuclei; in these the frequencies of positive micronucleate cells labeled in both main nuclei and micronuclei were 39.9 per cent (n = 163) and 42.6 per cent (n = 202), respectively. These values do not differ materially from those given in column 6 of this Table.

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to fixation, there were 119 labeled mitotic MN cells of which 30 contained labeled micronuclei; among these 30 cells, only 6 contained labeled micronuclei which had undergone mitotic chromosome condensation.

The size ranges of labeled and unlabeled micronuclei from both onion and bean roots, exposed to

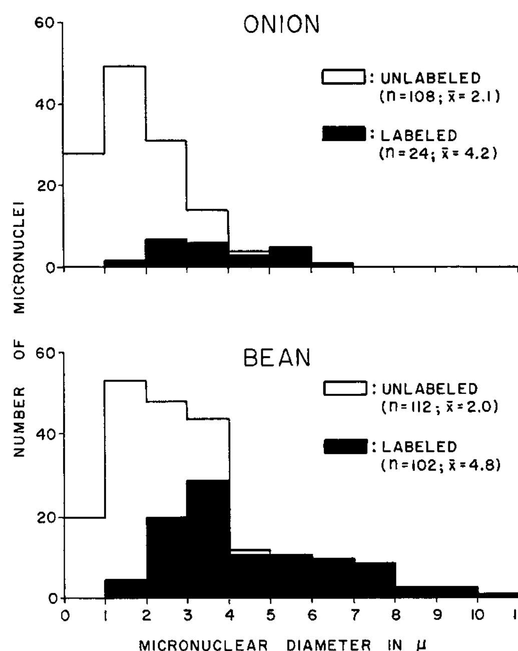

FIGURE 6

Size ranges of micronuclei, unlabeled and labeled after 4 hours' exposure to H^3-thymidine (2.0 μc/ml). Micronuclei were selected from cells in which main nuclei were labeled. Micronuclear diameters were measured on camera lucida drawings. Autoradiographs of Feulgen-stained squash preparations of 2 onion and 3 bean roots were exposed for 2 weeks. n = number; $\bar{x}$ = average diameter.

H^3-thymidine for 4 hours, are presented in Fig. 6. These micronuclei are taken only from cells in which the main nuclei are labeled. It can be seen in this figure that the average size of the labeled micronuclei is larger than that of the unlabeled ones but that many labeled micronuclei fall within the upper size range of the unlabeled ones. The size of a micronucleus does not necessarily reflect the amount of DNA it contains, since large micronuclei are often diffusely and lightly stained with Feulgen (Fig. 5). It thus appears that whether or not a micronucleus will synthesize DNA does not depend only on its size and initial DNA content, but also on the type of chromosome fragment from which it is derived.

The ability of a micronucleus to synthesize DNA can be correlated with the presence of persistent silver-stainable nucleolar bodies in it. Table II shows that in both onion and bean roots a very much greater proportion of labeled micronuclei contain nucleolar bodies than unlabeled micronuclei that are also selected from cells with labeled main nuclei (see also Figs. 2 *a* and *b*). The few unlabeled micronuclei which contain nucleolar material might have been exposed to thymidine before or after their period of DNA synthesis (for reports of asynchrony in DNA synthesis in chromosomes see Taylor, 1960; Wimber, 1961). Table II further shows that between 2 and 4 hours there is a decrease in the frequencies of micronuclei containing nucleolar bodies (both labeled and unlabeled). All micronuclei when they are first formed contain some nucleolar material, since all 58 chromosome fragments scored from telophase and all 170 micronuclei, irrespective of their sizes, from early interphase cells in these materials give a positive nucleolar reaction (*cf.* Das and Alfert, 1959; Das, 1962). A gradual loss of this material from some micronuclei appears to occur as interphase progresses.

TABLE II

Frequencies of Micronuclei, Unlabeled and Labeled with H^3-Thymidine (2 μc/ml), Containing Nucleolar Material

Exposure time to H^3-thymidine (26 hrs. after x-irradiation with 200 r)		Labeled micronuclei:		Unlabeled micronuclei:	
		Total	% with nucleolar material	Total	% with nucleolar material
	(*hrs.*)				
Onion	2	5	80.0	65	9.3
	4	19	73.6	88	4.5
Bean	2	88	90.9	185	3.8
	4	100	64.0	195	0.5

Micronuclei were selected from cells with labeled main nuclei. At each time 2 onion and 3 bean roots were used. Autoradiographs of Feulgen-stained squash preparations were scored after 2 weeks' exposure, and the nucleolar material in the same nuclei was detected by silver staining.

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(*b*) *RNA Synthesis:*

The data presented in Table III pertain to the frequencies of onion and bean micronuclei labeled after roots were exposed to H^3-cytidine for 1 hour and to H^3-thymidine for 2 hours (26 hours after x-irradiation with 200 r). Since RNA synthesis, and consequently the incorporation of cytidine, occurs at all stages of interphase, these frequencies are based on the total number of micronuclei

TABLE III

Frequencies/Root of Micronuclei Labeled with H^3-Cytidine and H^3-Thymidine

	Bean roots grown in:		Onion roots grown in:	
	H^3-cytidine for 1 hr.	H^3-thymidine for 2 hrs.	H^3-cytidine for 1 hr.	H^3-thymidine for 2 hrs.
Total micronuclei	974	991	721	341
per cent labeled micronuclei	16.7	16.2	5.8	0.3

Squash preparations (after pectinase digestion) from 3 onion and 3 bean roots, grown in H^3-cytidine (10 μc/ml), were treated with DNase and autoradiographs were obtained after 4 weeks' exposure; cells were stained with pyronin–Fast green through the processed film. The frequencies of micronuclei labeled with H^3-thymidine were determined from 2 onion and 3 bean roots grown in 0.5 and 2.0 μc/ml of H^3-thymidine, respectively; autoradiographs of Feulgen-stained onion and bean roots were exposed for 4 and 2 weeks, respectively.

scored. These data show that about 16 to 17 per cent of micronuclei in bean roots incorporate both H^3-thymidine and H^3-cytidine; on the other hand, about 6 per cent of onion micronuclei take up H^3-cytidine and only less than 1 per cent are labeled with H^3-thymidine (see also Figs. 7 *a* and 8). On the basis of continuous RNA synthesis in all interphase cells, as mentioned above, the frequencies of micronuclei labeled with H^3-cytidine would be expected to be higher than the frequencies of micronuclei labeled with H^3-thymidine during DNA synthesis. This is obvious in onion but not in bean. However, in both materials the frequencies of micronuclei labeled with H^3-cytidine are likely to be underestimated, because of the small amount of RNA, as compared to DNA, present in them, and also because of the presence of radioactivity in the cytoplasm which obscures the detection of specific micronuclear labeling.

Similar to the micronuclei that incorporate H^3-thymidine, the micronuclei that incorporate H^3-cytidine are on the average larger than those that fail to do so (Fig. 9). All these labeled micronuclei contain nucleolar material (Table IV; see also Figs. 7 *a* and *b*). Many of the unlabeled micronuclei that contain nucleolar material may be capable of undergoing RNA synthesis which, for the reasons mentioned previously, is not detected in the present experiment.

DISCUSSION

The results obtained from the incorporation of H^3-thymidine and H^3-cytidine in onion and bean root tip cells containing x-ray–produced micronuclei suggest that the onset of nucleic acid synthesis in micronuclei is under generalized cellular control (perhaps due to the availability of precursors; *cf.* Hotta and Stern, 1961), since those micronuclei which synthesize nucleic acid do so in synchrony with the main nuclei; no case was found in which a micronucleus incorporates H^3-thymidine or H^3-cytidine at a time when the main nucleus did not do so.

Most of the micronuclei present in x-rayed roots are likely to be derived from acentric chromosome fragments, because centric fragments have a better chance of being included in the nucleus during mitosis. A small fraction of micronuclei, however, may have originated from lagging chromosomes which are occasionally seen in such x-rayed materials. All these chromosome fragments and chromosomes at telophase exhibit silver-stainable nucleolar granules. (The silver stainability of these granules, as mentioned earlier, is due to a component other than nucleic acid). The present results show that in the subsequent interphase only some micronuclei can retain nucleolar bodies; others lose them as interphase progresses. In general, only those micronuclei which maintain nucleolar bodies undergo DNA and RNA synthesis; it seems, therefore, that the ability to synthesize nucleic acids is correlated with prolonged maintenance of nucleolar material. It may be postulated that the presence of discrete chromosomal sites (*cf.* Swift, 1959) is necessary for nucleic acid synthesis and for maintenance of nucleoli in micronuclei; only

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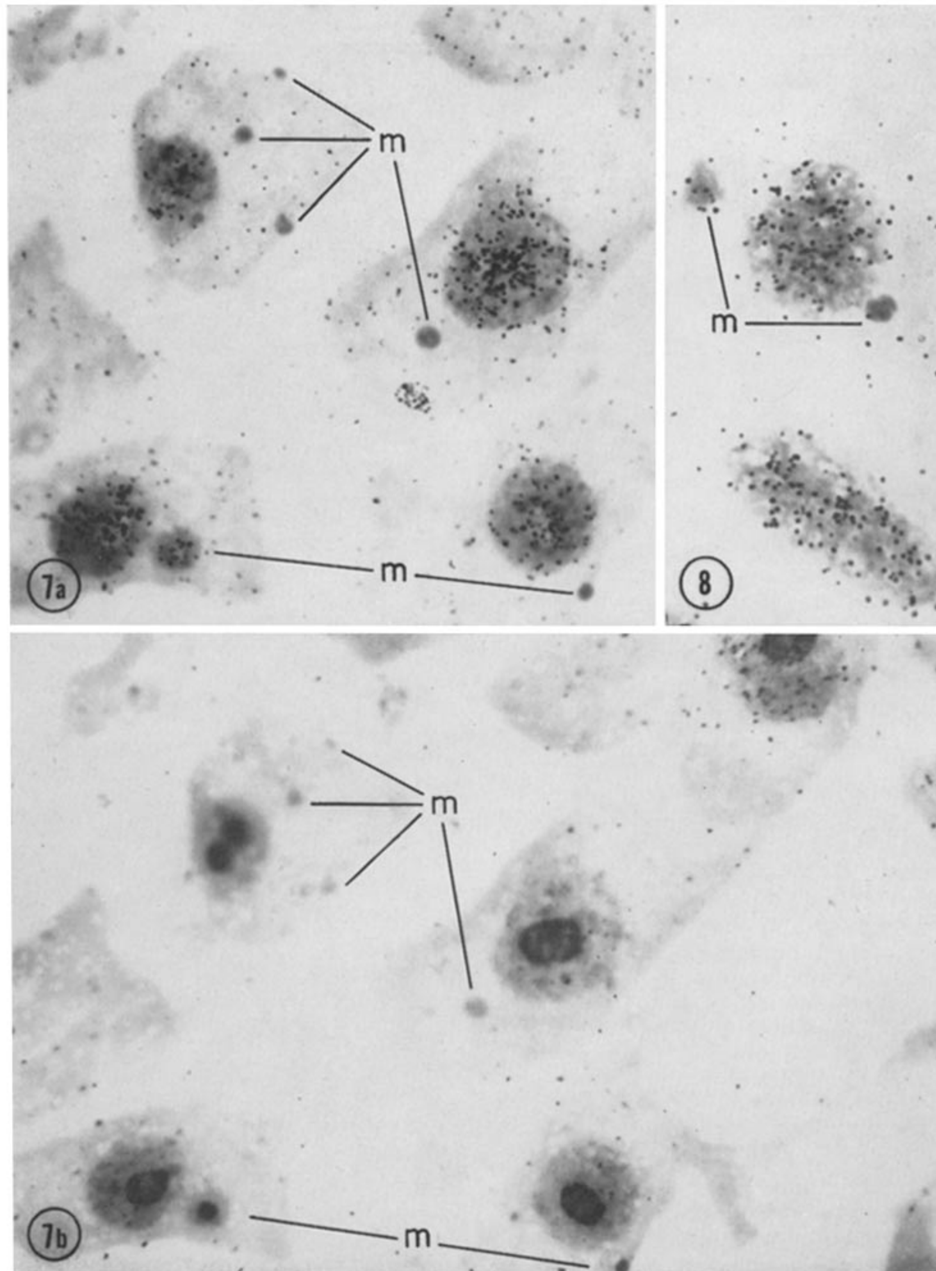

FIGURES 7 *a* AND 8

Autoradiographs of bean (Fig. 7 *a*) and onion (Fig. 8) root tip cells, exposed to H^3-cytidine (10 μc/ml) for 1 hour, showing unlabeled and labeled micronuclei (*m*) and labeled main nuclei. Roots were squashed following pectinase digestion, and autoradiographs were exposed for 4 weeks; nuclei were stained with methyl green through the processed film. Fig. 7 *a*, × 1200; Fig. 8, × 1270.

FIGURE 7 *b*

Silver staining of the same cells presented in Fig. 7 *a*; note the presence of nucleolar material only in the labeled micronucleus. × 1200.

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some of the chromosome fragments produced randomly by irradiation will contain one or more of these sites. The larger the fragment, the greater is the chance that it might contain such synthetically active sites. On the other hand, micronuclear size may, in turn, be determined by the production and accumulation of nucleolar

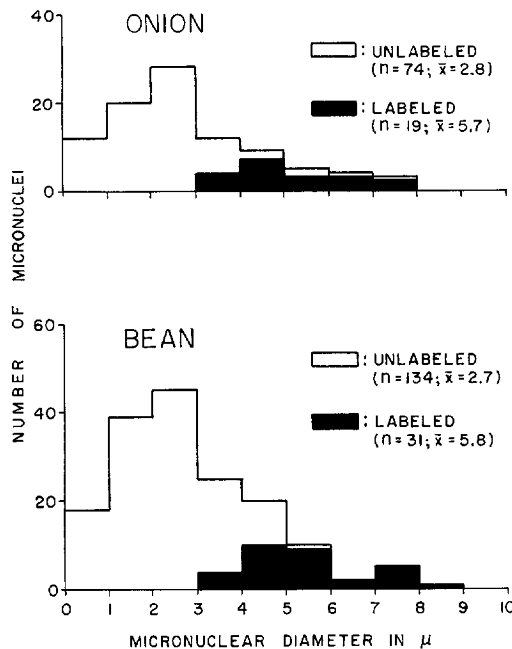

FIGURE 9

Size ranges of micronuclei, unlabeled and labeled after 1 hour's exposure to H^3-cytidine (10 μc/ml). Micronuclear diameters were measured on camera lucida drawings. Autoradiographs of squash preparations, following pectinase digestion, from 2 onion and 2 bean roots were exposed for 4 weeks. Cells were stained with methyl green through the processed film. n = number; $\bar{x}$ = average diameter.

substance. The absence of nucleolar material from some micronuclei labeled with H^3-thymidine (Table II) might be due to loss of this material following earlier cessation of the activity of these chromosomal sites.

It has been observed by La Cour (1953) and McLeish (1954) that in some plant materials, including bean, micronuclei derived from chromosome fragments enter mitosis along with the main nuclei if they contain nucleolar organizers (possibly also heterochromatin). For micronuclei, as for nuclei in general, DNA synthesis appears to be a necessary but not a sufficient prerequisite for entering mitosis; in the present study, only a few micronuclei labeled with H^3-thymidine are found to exhibit mitotic changes along with the main nuclei, while the unlabeled ones never do so.

Under similar experimental conditions, many more micronuclei in bean than in onion roots are found to be capable of nucleic acid synthesis. In both materials, the concentration of tritium per nucleus, as detected by over-all grain density, continues to increase between 2 and 4 hours' exposure to H^3-thymidine. Moreover, the total frequency of labeled MN cells increases at a much faster rate in onion than in bean roots

TABLE IV

Frequencies of Micronuclei, Unlabeled and Labeled after 1-Hour Exposure to H^3-Cytidine (10 μc/ml), Containing Nucleolar Material

Materials	Labeled micronuclei:		Unlabeled micronuclei:	
	Total	% with nucleolar material	Total	% with nucleolar material
Onion	19	100	73	27.4
Bean	54	100	276	6.5

Micronuclei were scored from squash preparations (after pectinase digestion) from 1 onion root and 2 bean roots. Nucleolar material was detected by the silver-staining technique after scoring the autoradiographs, which were exposed for 4 weeks. Nuclei were stained with methyl green before and after silver staining of nucleoli.

during these times (Table I, column 4). The lower frequency of positive micronuclei in onion, as compared to bean, is, therefore, not likely to be due to exhaustion of the precursor from the medium. Micronuclei from both materials fall within similar size ranges and, on the average, probably have similar DNA contents. Since a diploid onion nucleus contains about 1.5 times more DNA than a diploid bean nucleus (see Rasch and Woodard, 1959), the fraction of total nuclear DNA present in an average bean micronucleus is probably somewhat higher (at most 1.5 to 2 times) than that present in an onion micronucleus of similar DNA content. It would, therefore, appear likely that a bean micronucleus has a somewhat better chance to include chromosomal sites necessary for nucleolar maintenance and nucleic acid synthesis. However, such a consideration cannot explain the extreme discrepancy between the synthetic capacities of

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micronuclei in bean and onion; about 16 per cent of all micronuclei in bean and only 0.3 per cent in onion are found to synthesize DNA. Either these chromosomal sites are more numerous in the bean than in the onion complement, or those of the bean are less readily damaged by x-rays. The latter interpretation might also account for the considerable difference between the frequencies of labeled MN cells in onion and bean roots. (Unirradiated control roots of onion and bean have similar mitotic cycles and similar proportions of cells in DNA synthesis at any given time). Fundamental differences in the sensitivity of bean and onion chromosomes to acridine orange have recently been reported by Nuti-Ronchi and D'Amato (1961).

The necessity of nucleolar maintenance for RNA synthesis in micronuclei is obvious if one assumes that a major fraction of nuclear RNA synthesis may occur in the nucleoli themselves (Vincent, 1955; Brachet, 1957; Woods and Taylor, 1959; Fitzgerald and Vinijchaikul, 1959; McMaster-Kaye, 1960). It has also been observed that microbeam irradiation of nucleoli by ultraviolet rays (Perry and Errera, 1960) or by soft x-rays (Seed, 1960) leads to depression of nuclear RNA synthesis. Other workers claim that nuclear RNA synthesis originates in chromatin and that the nucleolus acts as a secondary center of RNA accumulation (Pelling, 1959; Goldstein and Micou, 1959; Feinendegen *et al.*, 1960; Rho and Bonner, 1961). Whether nuclear RNA synthesis occurs directly in nucleoli or at first in chromatin, all these studies point to the functional importance of nucleoli in cellular RNA metabolism.

The present results, which show that DNA synthesis also occurs only in those micronuclei which maintain nucleolar bodies, are compatible with those of some recent studies of cultured animal cells in which a rapid incorporation of H^3-thymidine was observed in chromosome regions associated with nucleoli (Chèvremont *et al.*, 1961). Since both RNA and DNA syntheses take place in those micronuclei which maintain nucleoli, and since onset of RNA synthesis precedes that of DNA, it is possible that the latter process is dependent on the former. In cultured connective tissue cells of rat, Harris (1959) observed that the synthesis of RNA and protein is a prerequisite for the synthesis of DNA. According to Feinendegen *et al.* (1961), a fraction of RNA may provide precursors for the synthesis of DNA in HeLa cells.

The present study reveals that the capacity of chromosome fragments (and chromosomes) to synthesize DNA and RNA, after they form micronuclei in the cytoplasm, is correlated with their ability to maintain nucleolar bodies. These results, therefore, support the concept that nucleoli and the specific chromosome sites with which they are associated are intimately involved in the primary biosynthetic processes of the cell.

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