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EARLY DNA SYNTHESIS DURING THE GERMINATION OF WHEAT EMBRYOS

BOZENA MARCINIAK, MARIA BUCHOLC and JERZY BUCHOWICZ

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; germination; DNA repair; DNA replication.

Abstract—Both [^{3}H]thymidine and [^{3}H]deoxyadenosine were found to be incorporated into the nuclear DNA of wheat embryos immediately after dry embryos were allowed to imbibe aqueous solutions of the radioactive precursors. The early labelled DNA sedimented in a manner suggesting that replicative intermediates were already formed within the first 90 min of germination. However, aphidicolin remained without any effect on this early DNA synthesis. Likewise, a cell-free system derived from early embryos incorporated [^{3}H]dCTP into DNA independently of the presence of aphidicolin. On the contrary, dideoxyTTP inhibited the DNA synthesis considerably. It is concluded that a proportion of the resting wheat embryo cells is able to initiate a replicative DNA synthesis immediately upon imbibition. The synthesis seems, however, to proceed with the participation of a γ-like, rather than an α-like, DNA polymerase.

INTRODUCTION

When dry wheat embryos are allowed to imbibe water, the synthesis of RNA and protein is rapidly activated, whereas the replication of DNA is resumed after a considerable delay (for a review, see ref. [1]). Poor incorporation of labelled thymidine during the early germination stages seems to indicate, however, that the first post-dormant round of DNA replication may be preceded by an unscheduled DNA repair synthesis. The use of [^{3}H]deoxyadenosine as the radioactive DNA precursor which, unlike [^{3}H]thymidine, is not diluted extensively with the endogeneous nucleoside, has shown that DNA synthesis is already unequivocally triggered within the first 30 min of imbibition [2].

The experiments described here are designed to elucidate the nature of the early DNA synthesis. To distinguish between the replicative and repair syntheses, wheat embryos were germinated in the presence of various inhibitors, with the use of either [^{3}H]thymidine or [^{3}H]deoxyadenosine as the radioactive precursor. In addition, the early labelled DNA was characterized by centrifugation in alkaline sucrose gradients. The results obtained seem to indicate that both repair and replicative events are triggered at the onset of germination.

RESULTS

The influence of selected inhibitors on the synthesis of nuclear DNA in wheat embryos at the initial and advanced stages of germination is shown in Table 1. Aphidicolin showed no effect on the incorporation of [^{3}H]thymidine at the initial germination stage (90-min embryos). At the advanced stage (16-hr embryos), the inhibition was significant but rather weak. Even an unusually high (300 μM) aphidicolin concentration reduced the incorporation of [^{3}H]thymidine only moderately. Dideoxythymidine triphosphate showed a considerable inhibitory effect both at the initial and advanced germination stages. The inhibitory effect of *N*-ethylmaleimide, α-amanitin and cycloheximide were also similar in both experiments. Factors that influenced the incorporation of [^{3}H]thymidine also reduced the increase in DNA content. Essentially the same results were obtained when [^{3}H]thymidine was replaced by [^{3}H]deoxyadenosine. In particular, the incorporation of [^{3}H]deoxyadenosine was inhibited by dideoxyTTP to the same extent as that shown in Table 1 for [^{3}H]thymidine. Neither of the inhibitors influenced the uptake of the radioactive precursors.

These observations indicate that early DNA synthesis, like DNA replication, depends on the synthesis of RNA and protein but, unlike replication, is not affected by aphidicolin. The aphidicolin effect was, however, unexpectedly weak even in the case of 16-hr embryos where, as shown by the increase in the nuclear DNA content (see Table 1), replication has already begun. It might therefore be assumed that the inhibitory effect of aphidicolin was limited by its uptake and penetration into the embryo cells. To eliminate such circumstances, a cell-free system was used in the next experiments.

The cell-free system prepared from either resting or germinating wheat embryos was able to incorporate [^{3}H]dCTP efficiently (Fig. 1), and that derived from 16-hr embryos was the most active (Fig. 2). Probably the 16-hr germination period corresponds to a germination stage at which a relatively high proportion of the embryo cells enters the S-phase. The inhibitory effect of aphidicolin *in vitro* was, however, again weak (Table 2). Under the same assay conditions, an authentic sample of DNA polymerase α was inhibited strongly by aphidicolin (89 and 95% inhibition for 60 and 300 μM aphidicolin concentrations, respectively; data not included in Table 2). The *in vitro* DNA synthesis was inhibited considerably by dideoxyTTP and ethidium bromide. The inhibition observed in systems derived from 90-min and 16-hr embyos was roughly the same. As summarized in Table 2, both

Table 1 Effects of various inhibitors on the incorporation of [^{3}H]thymidine into nuclear DNA by germinating wheat embryos

Germination time	Inhibitor	nDNA content (μg/embryo)	nDNA radioactivity (cpm/μg)	Inhibition (%)
90 min	None	5.5	45.0	–
	Aphidicolin, 60 μM	n.d.	45.6	< 5
	Aphidicolin, 300 μM	n.d.	45.2	< 5
	DideoxyTTP, 10 mM	n.d.	29.7	34
	NEM, 8 mM	n.d.	2.3	95
	α-Amanitin, 90 μg/ml	n.d.	20.2	55
	CHI, 50 μl/ml	n.d.	32.4	28
16 hr	None	6.4	72.5	–
	Aphidicolin, 60 μM	6.4	60.2	17
	Aphidicolin, 300 μM	6.2	44.9	38
	DideoxyTTP, 10 mM	6.0	37.9	51
	NEM, 8 mM	5.6	1.7	98
	α-Amanitin, 90 μg/ml	5.9	29.1	63
	CHI, 50 μg/ml	5.9	29.2	63

In each experiment, [^{3}H]thymidine (25 μCi/ml, 47 Ci/mmol) was present in the germination medium for the last 90 min of incubation. Inhibitors, when used, were present throughout the period investigated. Inhibition percentage is calculated from the total incorporation, assuming no changes in DNA content during the first 90 min of germination. Average values of triplicate determinations, differing by less than 10%, are given. Abbreviations: nDNA, nuclear DNA; n.d., not determined; NEM, *N*-ethylmaleimide; CHI, cycloheximide.

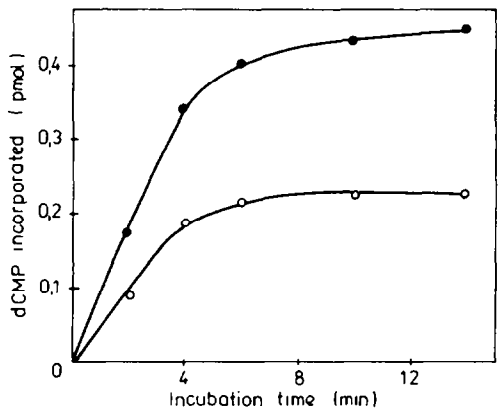

Fig. 1. Rates of [^{3}H]dCTP incorporation by cell-free systems prepared from resting (○) and 20-hr germinated (●) wheat embryos. The cell-free systems were prepared and incubated for the indicated time periods as described in the Experimental.

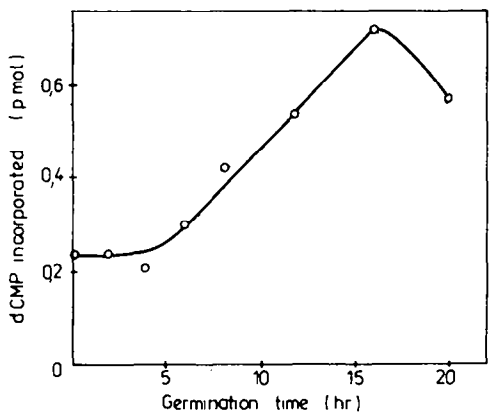

Fig. 2. Incorporation of [^{3}H]dCTP by cell-free systems prepared from wheat embryos germinated for various time periods. After germination for the indicated time, the embryo sample was harvested and used to prepare the *in vitro* assay system (see Experimental).

systems responded, in general, similarly to changes in the incubation conditions.

In an attempt to characterize the earliest products of post-dormant DNA synthesis, dry wheat embryos were imbibed for 30 min in a water solution of [^{3}H]deoxyadenosine. The nuclear DNA was then analysed by alkaline sucrose gradient centrifugation (Fig. 3a). The radioactive product sedimented together with the chromosomal size DNA mainly, suggesting that repair events, rather than replicative ones, contributed predominantly to the early DNA labelling. However, even 90 min after the beginning of imbibition, radioactive products of relatively low M_r appear in addition to the chromosomal size DNA (Fig. 3b). Peaks of radioactivity corresponding to the presumed replicative intermediates become even more prominent when the continuous labelling is replaced by pulse labelling conditions (Fig. 4a). Similar sedimentation patterns were found for pulse-labelled DNA from 9-hr (Fig. 4b) and 16-hr (not shown) embryos. Comparison of Figs. 4a and 4b may lead to the conclusion that early DNA synthesis is, in its nature, replicative to the same extent as that taking place at advanced germination stages.

Table 2. Effects of various factors on the incorporation of [^{3}H]dCTP by cell-free systems derived from germinating wheat embryos

Omissions or additions	dCMP incorporated (% of complete assay*) Expt 1	Expt 2
−dATP, dGTP, dTTP	68	64
−ATP	35	40
+ Aphidicolin, 60 μM	100	84
+ Aphidicolin, 300 μM	98	65
+ DideoxyTTP, 1 mM	75	67
+ DideoxyTTP, 10 mM	46	41
+ Ethidium bromide, 40 μM	69	60
+ KCl, 0.2 M	114	107
None, zero time	< 5	< 5

*0.25 and 0.72 pmol for experiment 1 (90-min embryos) and experiment 2 (16-hr embryos), respectively.

DISCUSSION

Replicative DNA synthesis at the onset of germination is somewhat unexpected since the first cell divisions in germinating wheat embryos cannot be observed earlier than 18 hr after the beginning of imbibition [3]. Limitations of the cytological methods used to complete the mitotic index data could, however, leave undetectable cell divisions in fewer than one case per 1000 cells. It should also be remembered that endoreplication of DNA, a replication that is not connected directly with cell divisions, occurs commonly in monocotyledonous plants [4]. In addition, wheat cell nuclei are known to contain extrachromosomal DNA [5], which may be thought to replicate independently of the cell divisions. The failure of previous attempts to detect early DNA synthesis might be due to the use of labelled thymidine at too low a radioactivity [3, 6–8] or at too low molar [9–13] concentrations.

Although replicative intermediates seem to appear within the first 90 min of germination, early DNA synthesis in wheat embryos is insensitive to aphidicolin, a specific inhibitor of DNA polymerase α. On the other hand, dideoxyTTP and ethidium bromide, factors that do not affect DNA polymerase α but strongly inhibit DNA polymerases β and γ (see ref. [14] and papers quoted therein), show inhibitory effects. In this connection, the recent findings of Graveline *et al.* [15] should be particularly considered. According to these authors, in wheat embryos, γ-like polymerase, rather than α-like polymerase, plays an essential role in the replicative, RNA-primed DNA synthesis. The acceptance of this view would allow reconciliation of our two apparently conflicting observations—the lack of an aphidicolin effect and the appearance of replicative intermediates at the early germination stages. However, further investigations are necessary to establish whether the early labelled polydeoxyribonucleotides represent typical replicative intermediates or autonomously replicating extrachromosomal DNA.

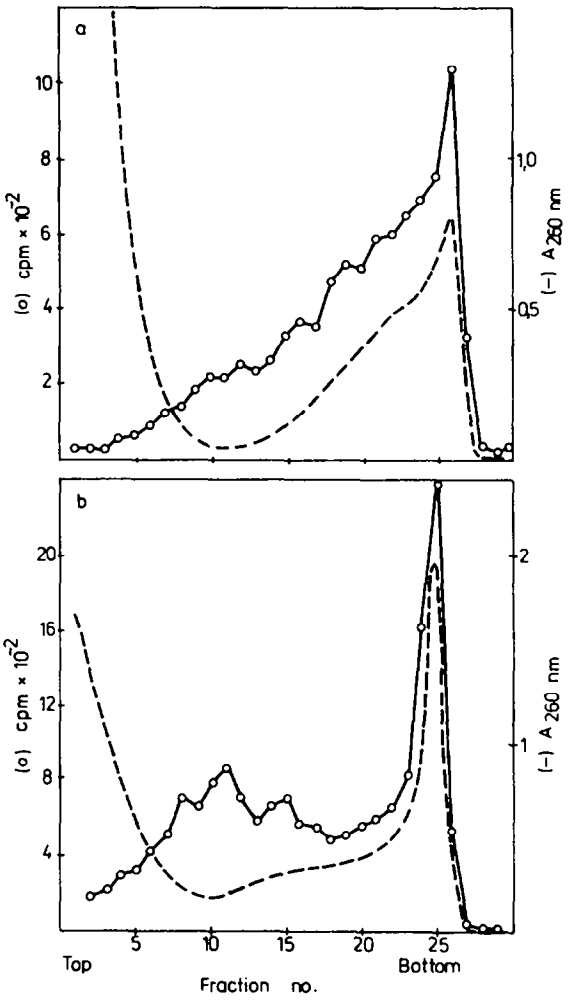

Fig. 3. Alkaline sucrose gradient sedimentation analysis of early labelled nuclear DNA from germinating wheat embryos. (a) Lysate of nuclear fraction (270 μg DNA, 9800 cpm) from embryos germinated for 30 min in the presence of [^{3}H]deoxyadenosine. (b) Lysate of nuclear fraction (420 μg DNA, 18 700 cpm) from embryos germinated similarly for 90 min. For germination, lysis and centrifugation conditions, see Experimental.

EXPERIMENTAL

Embryos. Dry grains of wheat (*Triticum aestivum* L. cv. Mironovska 808; post-harvest storage, 4–8 months) were ground in a Waring blendor and intact embryos were harvested manually. The isolated embryos showed a high viability (95% germination). They weighed, on average, 0.6 mg and contained 6.5 μg DNA/embryo. Changes in the DNA content during germination have been reported [2].

Germination and labelling conditions. Embryos were sterilized and germinated at 21° as described previously [2] using either [*methyl*-^{3}H]thymidine (47 Ci/mmol) or deoxy[G-^{3}H]adenosine (25 Ci/mmol) as radioactive precursor. Unless otherwise indicated, the precursors were given at a concn of 180 μCi/ml. Continuous or pulse labelling times, as well as the use of inhibitors, were as specified for each expt in the Results section (a stock soln of aphidicolin was made in DMSO). The precursor

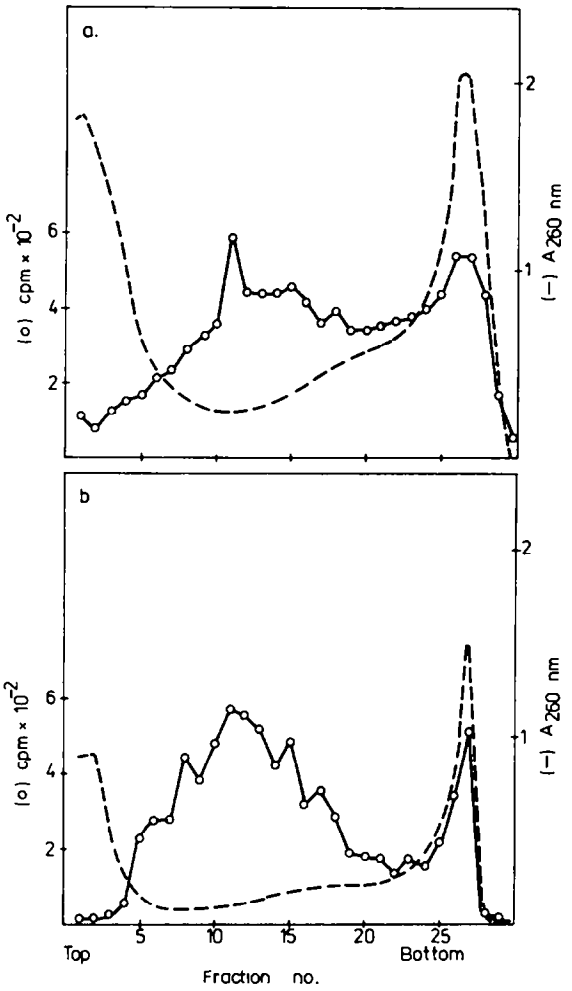

Fig. 4. Alkaline sucrose gradient sedimentation analysis of pulse labelled nuclear DNA from germinating wheat embryos. (a) Lysate of nuclear fraction (725 μg DNA, 10200 cpm) from embryos germinated for 90 min, in the presence of [^{3}H]deoxyadenosine during the last 30 min of germination. (b) Lysate of nuclear fraction (330 μg DNA, 8750 cpm) from embryos germinated similarly for 9 hr. For germination, lysis and centrifugation conditions, see Experimental.

uptake was measured as described in ref. [7] and was similar to that reported previously [2, 16].

Isolation of nuclear fraction. After germination, embryos were rinsed extensively with H_2O and ground using a pre-chilled pestle and mortar and ice-cold extraction buffer (300 mM sucrose, 50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.4). Usually, 6 ml of the buffer was used per 300-embryo sample. The homogenate was filtered through Miracloth and the resulting filtrate, after addition of Triton X-100 (1% final concn), was centrifuged at 4° for 6 min at 900 g. The nuclear pellet was resuspended in the extraction buffer and again centrifuged as above to obtain the nuclear fraction.

Alkaline lysis and sucrose density-gradient centrifugation. The nuclear fraction was lysed for 2 hr at 37° in an alkaline lysis soln (0.3 M NaOH, 0.3 M NaCl, 30 mM EDTA, 0.5% SDS) and carefully transferred onto the top of a linear 5–20% sucrose gradient (15 ml) containing 0.9 M NaCl, 0.3 M NaOH and 1 mM EDTA, with a 1 ml cushion of 2 M sucrose at the bottom. Sedimentation was at 24000 rpm for 17 hr at 21° in the SW 28.1 rotor of a Beckman model L ultracentrifuge. Fractions of 0.6 ml were collected from the top and assayed for DNA radioactivity by a modified filter paper disc method [2]. Positions of markers, T7 DNA (37S) and T4 DNA (73S), were found in a parallel expt. These peaked at fractions 14 and 20, respectively.

Assay for in vitro [^{3}H]*dCTP incorporation.* Preparation of the cell-free system and incubation conditions were essentially the same as those described for the lysate of S-phase HeLa cells [17]. However, [^{3}H]dCTP (4 μCi, 1.9 Ci/mmol) was used instead of [^{3}H]dTTP, and the incubation mixture (0.5 ml) contained the wheat embryo homogenate in an amount equivalent to 194 μg DNA. After incubation (10 min, 37°), the TCA-precipitable product was hydrolysed with 0.1 M HCl for 10 min at 100° to obtain DNA hydrolysate for the measurement of radioactivity. Under these conditions, a radioactivity of 1000 cpm corresponds to 0.4 pmol of dCMP incorporated.

DNA polymerase α was a kind gift from Dr. J. Siedlecki (Institute of Oncology, Warsaw). The preparation was isolated from calf thymus according to ref. [18]. The inhibitory effect of aphidicolin on the enzyme activity was tested in the complete *in vitro* assay system (see above).

DNA content and radioactivity were determined as described in ref. [2], except that the time of the hot acid treatment was always extended to 10 min. Average values of triplicate determinations, differing by less than 10%, are given throughout.

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