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DNA POLYMERASE ACTIVITY IN EMBRYOS OF *ALLIUM CEPA* SEED

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Abstract—A low level of DNA polymerase (deoxyribonucleosidetriphosphate: deoxynucleotidyltransferase; E.C. 2.7.7.7) activity can be detected by autoradiography after incubating frozen sections of unimbibed onion seed embryos in an aqueous solution of tritium labelled deoxynucleoside triphosphate. The enzyme is sensitive to sulfhydryl reagents, and incorporation was reduced by high levels of deoxyribonuclease, but inclusion of cycloheximide, ribonuclease, or nucleoside triphosphate did not change activity. On germination, activity remained relatively constant and at a low level until 30 hr after imbibition when it began to increase significantly.

INTRODUCTION

SEED germination is characterized by an initial synthesis of protein on preformed *m*RNA,[1-3] with subsequent protein synthesis on *m*RNA, transcribed from DNA after germination.[4-6] Incorporation of precursors into DNA in germinating seeds is preceded by amino acid incorporation by several hours.[7-9] In wheat grains, however, when presoaked at 2° and then restored to conditions favorable to germination, the initiation of protein and DNA synthesis nearly coincides.[10] Most evidence suggests that factors are regenerated which control the initiation of DNA synthesis. Although considerable progress has been made in investigations of the multiple DNA polymerases of prokaryotic cells, and more recently in eukaryotic cells, DNA polymerase activity in plant tissue is not well characterized.[11-13] Recent work[14] from this laboratory has demonstrated a high level of *in vitro* DNA polymerase activity in frozen sections of meristematic plant tissue which has been tentatively ascribed to a DNA polymerase involved in genuine DNA replication in *S* phase, as opposed to repair or recombinational synthesis. An attempt was made to investigate DNA polymerase activity in various tissues of unimbibed onion seed embryos by this method. *In vivo* ^{3}H-thymidine labelling can only be detected after *ca.* 35 hr imbibition, similar to results reported by Bryant.[15]

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RESULTS

A low level of DNA polymerase can be detected in the pre-emergent onion embryo. Activity is not confined to any particular tissue, but can be found in provascular, leaf primordium, cotyledonary and root cap zones. There is variability in nuclear labelling in different tissues and different embryos. Inclusion of *N*-ethylmaleimide or *p*-chloromercuribenzoate in the incubation mixture (minus dithiothreitol) reduced activity considerably (Table 1), whereas no significant incorporation was found in the presence of DNA^{se}. Similary slides treated with DNA^{se} effectively eliminated activity, whereas DNA^{se} was without effect. Inclusion of thymidinetriphosphate (TTP) alone in the incubation mixture considerably reduced labelling but did not eliminate it; it is not known if this is indicative of a low level of a terminal transferase activity. No increase in activity was obtained by including low concentrations of DNA^{se} in the incubation mixture; similarly the inclusion of ATP or GTP did not change activity. When cycloheximide or RNA^{se} was included in the incubation mixture, activity appeared to be unchanged. The low level of polymerase activity in the dry embryo can be contrasted with a much more intense labelling seen after *ca.* 35 hr when the cells enter *S* phase.

TABLE 1. EFFECT OF CHANGES IN REACTION MIXTURE ON DNA POLYMERASE ACTIVITY IN UNIMBIBED EMBRYOS

Reaction mixture	% Cells with label	Reaction mixture	% Cells with label
Complete	19*	+100 μg ml^{-1} RNA^{se}	16
+0·025 μg ml^{-1} DNA^{se} I	18	+2 mM *p*-chloromercuribenzoate	<2
+0·1 μg ml^{-1} DNA^{se} I	17	+2 mM *N*-ethylmaleimide	<2
+50·0 μg ml^{-1} DNA^{se} I	4	+1 or 3 mM Adenosine triphosphate	17
+50 μg ml^{-1} Cycloheximide	16	+1 or 3 mM Guanosine triphosphate	19
+10 μg ml^{-1} Cycloheximide	17		

The complete incubation mixture contained 0·05 M Tris-HCl (pH 8·3), 0·5 mM each of *d*CTP, *d*GTP, *d*ATP, 100 μCi ^{3}H-dTTP (18 Ci mM^{-1}), 5 mM $Mg(OAc)_2$, 10 mM KCl, 1 mM dithiothreitol

*Mean for approximately 10^5 cells from a random sample of 150 sections from 10 seeds per experiment. All experiments were performed in duplicate.

DISCUSSION

Recently several systems capable of carrying out semiconservative DNA replication have been described in which bacterial cells have been subjected to lysis with toluene.[16–20] It has often been shown that the replicative synthesis in these systems is dependent on ATP (or other nucleoside phosphates) and is sensitive to sulfhydryl blocking agents such as *N*-ethylmaleimide and *p*-chloromercuribenzoate, while the repair type synthesis is dependent on either endogenous or exogenous nuclease activity, is not semiconservative, ATP-stimulated, or sensitive to –SH blocking agents. Sulphydryl reagents have also been reported to inhibit certain types of DNA polymerase activities in eukaryotic

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cells.[21–23] In our system the sulphydryl reagents appeared to significantly reduce activity whereas inclusion of varying concentrations of ATP or DNA^{se} in the reaction mixture were without effect. A negative ATP effect in a toluene-treated system displaying replicative synthesis has been reported for the unicellular alga[24] *Chlamydomonas*, in contrast to comparable bacterial systems.

The problem of whether the native replicating polymerase acting in the cell uses as its immediate template single or double stranded DNA remains controversial. Inclusion of small concentrations of DNA^{se} in our system appeared not to increase activity. We have, however, demonstrated by scintillation counting from a high speed spin supernatant (unpublished results, Payne and Bal) an increased activity with DNA^{se}-treated[25] calf thymus DNA. Further investigations are necessary to determine if such activities are due to the same polymerase(s) as that being shown on autoradiographs. Much evidence has been presented[26–27] that protein synthesis is required for the initiation of chromosome replication. However, the ability to sustain DNA replication in the absence of protein synthesis has been shown to vary according to the system studied.[28–32] In an earlier study[33] of protein metabolism during *Allium* germination, Mallery has shown a markedly low level of ^{3}H-leucine incorporation before 30–36 hr, at which time activity dramatically increased. Our autoradiographs for *in vivo* labelling with ^{3}H-amino acids gave a similar result. The demonstration of a similar activity in our system with the use of cycloheximide and RNA^{se} suggests that newly translated protein is not required to initiate or sustain the DNA polymerase activity found here.

EXPERIMENTAL

Frozen sections were prepared with a cryostat[34] except that sections were not fixed to microscope slides, but were placed directly in a small vol. of the incubation mixture. We have observed that such sections retain structural integrity in hypotonic media; once growth is initiated, however, there is a much greater tendency for cells to rupture. Embryos were individually dissected from dry seeds with the aid of microscope. For each experiment 7–10 dissected embryos were frozen in microtome chucks and sectioned at 8–10 μm; *ca.* 150 sections were collected and placed with the embedding gel intact into test tubes containing 0·5 ml of reaction mixture. The incubation mixture contained 0·05 M Tris-HCl (pH 8·3), 0·5 mM each of *d*CTP, *d*GTP, *d*ATP, 100 μCi ^{3}H-*d*TTP (18 Ci mM^{-1}), 5 mM Mg $(OAc)_2$, 10 mM KCl, 1 mM dithiothreitol. EtOH was removed from ^{3}H-TTP under N_2 before use. Besides its effect on enzyme inhibition, the EtOH was also observed to precipitate the embedding gel in soln. Before sectioning, excess embedding gel was trimmed; the effect of the gel on enzyme activity has not been investigated. Reactions were terminated with 45% HOAc, 50 mM $Na_4P_2O_7$ after 1 hr incubation at 32°. After termination of the reaction, sections were centrifuged at 500 *g* to remove the incubation mixture, washed several times with cold trichloracetic acid, 95% EtOH and ethyl ether. Sections were finally suspended in 50% EtOH and spread on microscope slides with a Pasteur pipette.

In some experiments an attempt was made to inhibit protein synthesis with cycloheximide. In preliminary experiments the efficiency of cycloheximide as an inhibitor of protein synthesis in the actively growing root meristem was tested. Whole roots (7 mm long) were exposed to ^{3}H-leucine or ^{3}H-leucine + 25 μg ml^{-1} of

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cycloheximide in 0·05 M Tris-buffer pH 7 3 for 1 hr. Grain count was reduced in the cycloheximide treated meristems by >90%. In another set of experiments ribonuclease was used at a concentration of 100 μg ml^{-1} to destroy any functioning translational system present in the dry seed. The sulfhydryl inhibitors *N*-ethylmaleimide and *p*-chloromercuribenzoate were used at a concentration of 2 mM.

In an attempt to activate the genome, concentrations of DNA^{se} from 25 ng ml^{-1} to 50 μg ml^{-1} ($\sim$135 units) or alternatively 1–3 mM ATP or other nucleoside triphosphates were included in the incubation mixture. Attempts to make permeable small slices of tissue with toluene[16–20] or sucrose,[35] instead of freeze sectioning, were tried but abandoned because of lack of activity.

Besides the inclusion of DNA^{se} in the incubation mixture, slices were treated with DNA^{se} (E.C. 3.1.4.5) or RNA^{se} (E.C. 2.7.7.16) (100 μg of DNA^{se} or RNA^{se} ml^{-1} in 0 05 M Tris-HCl pH 7·2, 5 mM $Mg(OAc)_2$, 5 mM KCl).

To investigate the initiation of protein and DNA synthesis *in vivo*, seeds were hydrated and at varying times a portion of the seed coat was removed exposing the embryo and either ^{3}H-leucine or ^{3}H-arginine (25 μCi ml^{-1}; sp. act. 890 mCi mM^{-1}) for protein synthesis and ^{3}H-thymidine (25 μCi ml^{-1}, sp. act. 6·7 Ci mM^{-1}) for DNA synthesis, was added. Radioautographs were prepared as usual [34]

Nuclei were considered to demonstrate DNA polymerase activity if they contained at least twice as much label as the cytoplasm. *Ca.* 22% of the unimbibed seeds tested contained sufficient activity for analysis. There was no indication of premature germination in the seed stock which was >90% viable, after storage for approximately 3 yr. Radiochemicals were obtained from New England Nuclear, nucleotides from Sigma and Calbiochem, and enzymes from Worthington.

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