De Novo Biosynthesis of Deoxyribonucleic Acid Polymerase during Wheat Embryo Germination

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ABSTRACT
An 8-fold enhancement in the activity of a DNA-dependent DNA polymerase was found in extracts from germinating wheat (Triticum vulgare var. Florence) embryos, as compared to the activity found in extracts from ungerminated embryos. The enhancement of this activity during the first hours of germination is concomitant to the increase of a DNase activity. The two activities could be separated and the increased level of the DNA polymerase upon germination was observed in an enzymatic fraction which contains very low DNase activity. Addition of the protein synthesis inhibitor, blasticidin S, to germinating wheat embryos, reduced the increase in DNA polymerase. Incorporation of radioactive amino acids into a phosphocellulose preparation, which contains the DNA polymerase, starts during the first 6 hours of germination. The amount of radioactivity incorporated is doubled in the next 6 hours, and the incorporation is continued between 12 and 18 hours of germination.

Seed germination transfers the wheat embryo from the latent stage to an active developmental stage and triggers a series of molecular events. Protein synthesis starts 30 min after germination is initiated by water imbibition (9). On the other hand, active DNA synthesis cannot be observed before 15 hr of germination (1). The sequence of these two events suggested that early synthesized proteins may be a prerequisite for the subsequent DNA replication. It was shown that the onset of DNA replication in germinating wheat embryos depends on proteins synthesized before 9 hr of germination (12). One protein was investigated, and it was observed that the DNA-dependent DNA polymerase activity present in crude extracts of wheat embryos is 8-fold enhanced upon germination (13). In the present paper, we report some evidence that the enhancement of the DNA polymerase activity is dependent on de novo protein synthesis and that the enzyme itself is synthesized de novo during germination.

MATERIALS AND METHODS
Chemicals. Radioactive amino acids were purchased from the Radichemical Center, Amersham; radioactive and nonradioactive deoxynucleotide triphosphates and ammonium sulfate (enzyme grade) from Schwarz-Mann; yeast β-nicotine adenine dinucleotide, yeast alcohol dehydrogenase, BSA (fraction V), crystallized and lyophilized ovalbumin, bovine pancreas α-chymotrypsinogen-A, and horse heart cytochrome-c, from Sigma Chemical Corp; acrylamide, N,N',N'-tetramethyl-ethylenediamine from Eastman; calf thymus DNA, salmon testes DNA, and deoxyribonucleic I from Worthington Biochemical Corp. GF/C filters were purchased from Whatman. Blasticidin S was a gift of Dr. J. Noguchi, Kyoto University.

Radioactivity was measured in the Packard Tri-Carb scintillation counter in a standard toluene scintillator, when the samples were layered on filters, or in a standard dioxane scintillator, when the samples were directly counted.

Germination of the Embryos. Wheat embryos were prepared from Triticum vulgare var. Florence (purchased from the Experimental Station, Neve Yaar, Israel), according to the procedure of Johnston and Stern (4). The embryos were germinated on 1% (w/v) agar containing 2% sucrose in 6-cm Petri dishes. Germination was initiated by imbibing 500 mg of embryos with 1 ml of 2% sucrose solution.

Purification of Wheat Embryo DNA. Wheat embryo DNA was prepared according to the chloroform-phenol procedure of Marmur (10) and further purified according to the method of Chen and Osborne (1). 14C-DNA (300 cpm/μg) was prepared by the same procedure from embryos that had been germinated for 24 hr in the presence of 14C-thymidine (20 μCi/ml; 58 mCi/mmol).

Activation of Calf Thymus DNA. Activated calf thymus DNA was prepared by a partial digestion of DNA with pancreatic deoxyribonuclease according to the procedure described by Loeb (7) using a DNAse concentration 103 times higher than the one reported by Loeb.

Assay of DNA-dependent DNA Polymerase. The standard reaction mixture in a final volume of 0.25 ml contained 20 mM tris-HCl, pH 7.6; 4.4 mM MgCl2; 16 mM KCl; 0.1 mM of each dATP, dCTP, dGTP, dTTP; 1 μCi of 3H-TPP (14.5-19.2 Ci/mole), 4% (w/v) glycerol; 0.4 mM 2-mercaptoethanol; 45 μg of alkali-denatured, ungerminated wheat embryo DNA and the indicated amount of protein extract for each experiment. When assaying the phosphocellulose column and the glycerol gradient fractions, wheat DNA was substituted for by 50 μg of activated calf thymus DNA. Incubation was carried out at 35 C. The reaction was stopped by adding 1 ml of carrier salmon testes DNA solution (100 μg/ml), 0.5 ml of a saturated solution of Na2PO4, 0.5 ml of 20% (w/v) trichloroacetic acid, and 4 ml of 5% (w/v) trichloroacetic acid at 0 C. The precipitate was collected by suction onto GF/C filters. The filters were washed with 25 ml of cold 5% trichloroacetic acid, 10 ml of ethanol-ether (3:1, v/v) and 5 ml of ether, dried and counted by liquid scintillation.

Protein concentration was determined according to the method of Lowry (8) with BSA as a standard.

Assay of Deoxyribonuclease Activity. DNase activity was assayed by measuring the amount of added wheat embryos 14C-DNA rendered acid-soluble. The incubation mixture contained, in a final volume of 0.2 ml, the following: 20 mM tris-HCl (pH 7.6), 5% (w/v) glycerol, 5 mM KCl, 5 x 10-1 mM MgCl2, 5 x 10-1 M-mercaptoethanol, and 10 μg of 14C-DNA (320 cpm/μg).
Incubation was for 30 min at 35 C. The reaction was stopped, and the radioactivity present in acid-insoluble material was measured as described for the DNA-dependent DNA polymerase assay.

Assay of Alcohol Dehydrogenase Activity. Alcohol dehydrogenase activity was assayed by measuring the rate of NADH formation according to the method of Vallee and Hoch (16).

**Glycerol Gradient.** The molecular weight of the DNA-dependent DNA polymerase in a crude enzymatic preparation was estimated by its velocity sedimentation through glycerol gradients. Linear 10 to 30% glycerol gradients, in a volume of 4.8, were formed in cellulose nitrate tubes. They contained 20 mm tris-HCl (pH 7.6), 10 mm KCl, 1 mm MgCl₂, 1 mm 2-mercaptoethanol, and 1 mg/ml BSA. Samples of 0.2 ml containing a crude preparation of wheat embryos DNA-dependent DNA polymerase, yeast alcohol dehydrogenase and 5% glycerol were layered on the top of the gradients. The gradients were centrifuged for 8 hr at 45,000 rpm and 4 C in SW50 1 rotor of the Beckman ultracentrifuge. Fractions of 9 drops were collected. Determination of the molecular weight was made by measuring the mobility on SDS' gels relative to proteins of known molecular weight and by plotting mobilities against the logarithm of the molecular weight (15).

**Preparation of Enzymatic Fractions.** The preparation of crude extract, S-100, and fraction II was carried out as previously reported (13).

**RESULTS**

Enhancement of Deoxynucleotide Triphosphate Polymerase and Deoxyribonuclease Activities in Germinating Wheat Embryos. In a previous paper (12), we reported that at the time at which wheat embryos enter the S phase, the DNA-dependent DNA polymerase activity is increased 8-fold as compared to the activity of ungerminated embryos. In crude extracts derived from germinating embryos, the polymerase activity is found together with a deoxyribonuclease activity. Since the polymerase activity is more pronounced with “activated” calf thymus DNA than with denatured or native wheat embryo DNA (unpublished results), it can be suggested that this increase in polymerase activity, after germination has started, is somehow due to the DNAse activity which in vitro would activate the DNA as template by partial digestion, as in the case of E. coli DNA polymerase (5) and of HeLa cells DNA polymerases (17) which are much more active with activated templates than with native or denatured templates. We therefore followed the activity level of the two enzymes during the early stages of germination. As shown in Figure 1, in the crude extract derived from germinating embryos, the two activities increase in a parallel way.

**Separation of Polymerase and DNAse Activities.** In order to separate the polymerase from the deoxyribonuclease, the S-100 was fractionated by (NH₄)₂SO₄ precipitation (Fig. 2). The polymerase activity is recovered in the bottom of the tube and the ADH- and DNA-dependent DNA polymerase of each fraction were assayed.

From preliminary experiments, we observed that the viscosity and the density of 10 to 30% linear glycerol gradients are such that there is a linear relationship between time of centrifugation and distance traveled from the meniscus of the gradient by the wheat embryo DNA polymerase and the yeast ADH. Such a linear relationship is theoretically expected for sedimentation of macromolecules of the same specific volume in uniform media and was experimentally observed by Martin and Ames (11) in 5 to 20% linear sucrose gradients. Therefore, the sedimentation constant of the DNA-polimerase could be calculated as it is done in 5 to 20% sucrose gradients (11). Once the sedimentation constant of the DNA-dependent DNA polymerase is estimated, the molecular weight can be calculated according to the following expression (14):

$$S₁ = \left(\frac{MW₁}{MW₂}\right)^{1/3}$$

1 Abbreviation: SDS: sodium dodecyl sulfate; GM: germinating medium, standard 2% sucrose solution.
where $S$ designates the sedimentation coefficient and $MW$ the molecular weight.

**Polyacrylamide Gel Electrophoresis.** High pH polyacrylamide gels were prepared according to the method described by Davis (2) in 0.6-cm diameter tubes. The gel length was 6.5 cm. Electrophoresis was carried out in the cold at 2.5 mamp/gel, at constant current.

SDS gels were prepared according to the procedure of Laemmli (6) in 0.6-cm diameter tubes. Electrophoresis was carried out at room temperature at 4 mamp/gel.

In both cases, electrophoresis was stopped when the bromophenol blue dye had reached approximately 0.5 cm from the end of the gel. The gels were fixed and stained overnight in 0.2% Coomassie brilliant blue dissolved in 50% methanol-7% acetic acid. Destaining was carried out in 50% methanol-7% acetic acid.

The fraction that precipitates between 0 and 30% of (NH$_4$)$_2$SO$_4$ saturation, the fraction which precipitates between 30 and 50% (NH$_4$)$_2$SO$_4$ saturation, and the remaining supernatant are respectively referred to as fractions I, II, and III.

Fractions II, devoid of deoxyribonuclease activity, were prepared from ungerminated and germinated embryos, and the polymerase activities of the two fractions were compared (Table I). In Fraction II prepared from 18-hr germinating wheat embryos there is a 8.5-fold enhancement in polymerase activity as compared to the activity of fraction II derived from ungerminated embryos. Therefore, it can be concluded that the increase in DNA polymerase upon germination is independent from the concomitant increase in deoxyribonuclease activity. Table I shows also that upon inhibition of protein synthesis by addition of blasticidin S, the specific activity of DNA polymerase in fraction II is enhanced only 2.3 times. This means that upon inhibition of total protein synthesis during germination, the increase of the DNA polymerase is also markedly reduced. This result is of particular interest, since it was shown (12) that when protein synthesis is blocked by blasticidin S, DNA synthesis is inhibited, suggesting that a *de novo* synthesis of the enzyme or of any other protein factor involved in the DNA polymerase reaction is required for normal DNA synthesis.

**Effect of S-100 Derived from Ungerminated Embryos on DNA Polymerase Prepared from Germinating Embryos.** The low level of DNA polymerase in ungerminated wheat embryos may be due to an inhibitor which is inactivated upon germination. To verify this possibility, the effect of S-100 prepared from ungerminated embryos on the DNA polymerase activity of the S-100 prepared from germinating embryos was tested.

S-100 from ungerminated and from 18-hr germinating wheat embryos were prepared from lots of 500 mg of embryos. Samples (112 mg of protein per test tube) of S-100 prepared from germinating embryos were incubated for 60 min in standard DNA polymerase reaction mixture containing increasing amounts of S-100 prepared from ungerminated embryos (Table II). Up to a concentration of 103 mg/ml of protein per test tube (about 1:1 ratio), the S-100 from ungerminated embryos does not inhibit the DNA polymerase activity of the S-100 prepared from germinating embryos. Therefore, we may conclude that the S-100 of ungerminated embryos does not contain an inhibitor of the DNA polymerase, unless we assume that the inhibitor is present in a 1:1 or lower molar ratio with the DNA polymerase enzyme.

**Purification of DNA Polymerase.** Wheat embryo DNA polymerase is adsorbed by phosphocellulose, whereas the bulk of the proteins are not adsorbed. Therefore, we purified the enzyme by chromatography on phosphocellulose columns. Phosphocellulose was washed with 1 n HCl in 50% (v/v) ethanol, H$_2$O until neutral, 1 n KOH, and finally H$_2$O until neutral. A column (3.14 cm$^2$ x 7 cm) was prepared and equilibrated with GB buffer made 0.1 M in KCl. Because the enzyme seemed to be unstable at low protein concentrations, BSA (1 mg/ml) was included in the wash and elution buffers in order to obtain the highest recovery of activity. The column was loaded with 31 mg of fraction II proteins and eluted with a three-step gradient (Fig. 3).

At each step, one peak of protein was eluted. Peak 1 contains the bulk of proteins but almost no DNA polymerase activity. Peak 2 contains some protein and very low DNA polymerase activity. Peak 3 contains a very low amount of protein and most of the DNA polymerase activity.

**Purity of Phosphocellulose Peak 3.** In order to establish the degree of purity of the DNA polymerase, we analyzed it by gel electrophoresis. When we used non-SDS 7.5% gels overlayed with 0.2 ml of 3% gels, we could identify only one band that does not enter the 7.5% gel, although it migrates through the 3% layer. A similar result was obtained by using 5% gels overlayed with 3% gels. Analysis of peak 3 on SDS gels indicates that peak 3 contains, besides the BSA and other impurities that are introduced with the buffer, only one band (Fig. 4B). The molecular weight of this protein was estimated, in four different experiments on 10% SDS gels and in two different experiments on 14% SDS gels, to be 17,000 ± 1,600.

In order to establish if peak 3 contains only one band and that no other protein is hidden behind the BSA bands, we prepared a phosphocellulose column and we ran it in the same conditions described under Figure 3, omitting BSA from the buffers. In this way, we obtained a phosphocellulose peak 3 in which we could not detect any DNA polymerase activity although it should contain such activity. Analysis of this peak by electrophoresis on 10% SDS gels indicates that, in peak 3 no other protein except one with molecular weight 16,000 ± 350 could be detected (Fig. 5).

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**Table I. Effect of Blastidicin S on the Enhancement of$^3$HTTP Incorporation by Extracts Derived from Germinating Embryos**

The reaction was started by adding the following protein amounts per reaction mixture: fraction II from embryos germinated without blasticidin, 52 μg; fraction II from ungerminated embryos, 52 μg; fraction II from embryos germinated in the presence of blasticidin S, 43 μg.

<table>
<thead>
<tr>
<th>Protein conecn</th>
<th>Specific radioactivity</th>
<th>Protein conecn</th>
<th>Specific radioactivity</th>
<th>Protein conecn</th>
<th>Specific radioactivity</th>
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<tr>
<td>mg/ml</td>
<td>cpm/60 min/mg protein</td>
<td>mg/ml</td>
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<td>mg/ml</td>
<td>cpm/60 min/mg protein</td>
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<tr>
<td>Ungerminated Embryos</td>
<td>18-hr Germinated Embryos Without Blastidicin S</td>
<td>18-hr Germinated Embryos 20 μg/ml Blastidicin S</td>
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<tr>
<td>Fraction I</td>
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<td>22,495</td>
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**Table II. Effect of S-100 Prepared from Ungerminated Embryos on Deoxynucleotide Triphosphate Polymerase of S-100 Prepared from Germinating Embryos**

The deoxynucleotide triphosphate polymerase activity of S-100 prepared from germinated embryos was assayed in a reaction mixture that contained in 0.250 ml, besides the standard constituents, the indicated amounts of S-100 proteins derived from ungerminated embryos. Each reaction mixture contained 112 μg of S-100 proteins from germinated embryos.

<table>
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<th>Protein from Ungerminated Embryos</th>
<th>HTTP Incorporation</th>
</tr>
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<tr>
<td>μg</td>
<td>cpm/60 min/reaction mixture</td>
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<tr>
<td>13</td>
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Incorporation of Radioactive Amino Acid into Phosphocellulose, Peak 3. Since phosphocellulose, peak 3, contains only one protein, as estimated by gel electrophoresis, and since this peak contains most of the DNA polymerase activity, the incorporation of radioactive amino acids into peak 3 would be a measure of the de novo biosynthesis of the DNA polymerase. Three equal groups of embryos, 2 g each, were germinated in the conditions described under Table III. The first group was germinated from 0 to 6 hr of germination in standard GM supplemented with radioactive amino acids. The second group was germinated from 0 to 6 hr in standard GM and then transferred for an additional 6 hr in GM supplemented with radioactive amino acids. The third group was germinated from 0 to 12 hr in standard GM and then transferred for 6 hr in GM supplemented with radioactive amino acids. After the 6 hr labeling period a crude extract, an S-100, and a fraction II were prepared from each group of embryos according to the procedure previously described. Two ml of each fraction II were loaded on phosphocellulose columns, and the columns were eluted in the same conditions as described under Figure 3. Radioactivity, although at a very low level, appears into peak 3 already when the embryos are labeled between 0 and 6 hr and increases when labeling is done between 6 and 12 hr and it is still incorporated into peak 3 proteins when labeling is done between 12 and 18 hr of germination. Since the amount of radioactivity loaded on each column is different, this result might not express a real enhancement of radioactivity into peak 3. However, if we

![Fig. 3. Chromatography on phosphocellulose of fraction II proteins. A phosphocellulose column was prepared as described in the text. A sample of 8 ml of fraction II (31 mg of protein) was loaded at a flow rate of 22 ml/hr. Then, the flow rate was adjusted to 46 ml/hr, and the column was washed first with 39.6 ml of GB buffer made 0.1 M in KCl, then with 50.4 ml of GB buffer made 0.25 M in KCl, and finally with 69 ml of GB buffer made 0.7 M in KCl. All steps were carried out at 4 C. Fractions of 3.6 ml were collected, and samples of 10 ml of each fraction were assayed for DNA polymerase activity with 50 µg reaction mixture of activated calf thymus DNA. Absorbance at 280 nm was recorded against a blank that contained 1 mg/ml BSA. DNA polymerase (●); O.D. 260 (○).](https://www.plantphysiol.org/doi/10.1104/pp.55.4.440)

![Fig. 4. Electrophoresis on 10% SDS polyacrylamide gels. A: Elution buffer containing BSA, 1 mg/0.1 ml; B: phosphocellulose peak 3 prepared with BSA, 0.1 ml; C: phosphocellulose peak 3 prepared with BSA + reference proteins (10 µg each).](https://www.plantphysiol.org/doi/10.1104/pp.55.4.440)

The molecular weight of the native and active enzyme has been calculated by velocity sedimentation through glycerol gradient, using an internal marker yeast alcohol dehydrogenase, S0, w 7.6S (13); molecular weight 150,000 (3) (Fig. 6), and it was estimated to be 238,000. In other experiments not reported here, the molecular weight of wheat DNA polymerase relative to lactate dehydrogenase was estimated to be 210,000 in low salt gradients and 232,000 in high salt (1 M KCl) gradients.

![Fig. 5. Electrophoresis on 10% SDS polyacrylamide gels. A: A phosphocellulose column was prepared, loaded, and eluted as described under "Materials and Methods," except that BSA was excluded from all buffers. Peak 3 was concentrated five times and 0.1 ml were loaded on 10% SDS polyacrylamide gels. B: Phosphocellulose peak 3, prepared as in A, plus 10 µg of each reference protein (I: BSA; II: ovalbumin; III: chymotrypsinogen; IV: cytochrome C).](https://www.plantphysiol.org/doi/10.1104/pp.55.4.440)
centrifuged the Beckman ultracentrifuge. Activity of tris-HCl (pH A) might be incorporated into the polymerase. That calculation of dependent activity that ranged from 0 to 6 hr in standard GM and then from 6 to 12 in GM supplemented with radioactive amino acids as a. (c) The third group was germinated from 0 to 12 hr in standard GM and from 12 to 18 in GM supplemented with radioactive amino acids as in a. After the 6-hr labeling period a crude extract on S-100 and a fraction II were prepared from each group of embryos. Those three fractions II were prepared by precipitating with (NH₄)₂SO₄, 23 ml of S-100 and by resuspending the pellet in 8 ml of GB-buffer. Two ml of each fraction II were loaded on phosphocellulose columns (3.14 cm² x 7 cm), and the columns were eluted as described under Fig. 3. Samples of all fractions were dissolved in a dioxane scintillation mixture and counted for radioactivity. The total radioactivity present in each phosphocellulose peak 3 was calculated. Two experiments were carried out independently and gave almost the same result.

**DISCUSSION**

The possibility that the availability of an active DNA polymerase might be one of the limiting steps for the entrance of wheat embryos into the S phase was suggested by the fact that this activity is greatly enhanced upon germination (12). Evidence was given that wheat embryo DNA polymerase synthesizes in vitro DNA-like material on a DNA template (13).

When wheat embryos were germinated in the presence of blastocidin S, the enhancement of DNA polymerase activity was markedly reduced. Although this is not a definite proof, it supports our suggestion that an active DNA polymerase might be required for the onset of DNA replication.

The enhancement of the DNA polymerase is paralleled by the enhancement of a deoxyribonuclease activity (Fig. 1). This enhancement suggests that the increase in DNA polymerase activity is dependent on the activity of the deoxyribonuclease, which is known to activate DNA templates (5, 17). This possibility was ruled out by separating the two activities (Fig. 2) and showing that the DNA polymerase activity of fraction II, devoid of deoxyribonuclease, is enhanced upon germination (Table I).

The basis of our experiments, we could not identify in the dry embryos any DNA polymerase inhibitor, unless we assume that such an inhibitor is present in a 1:1 molar ratio with the DNA polymerase. The presence of an unbound low molecular weight inhibitor is also unfavorable since the extract was extensively dialyzed before assaying it for DNA polymerase activity.

Purification of the wheat DNA polymerase was achieved by chromatography on phosphocellulose column. In this way, we obtained a DNA polymerase preparation which, as judged by electrophoresis on polyacrylamide gels, was devoid of most of fraction II proteins. We cannot, however, exclude the presence in this preparation of other minor bands which could not be stained.

Radioactive amino acids are already incorporated in the phosphocellulose preparation in the first 6 hr of germination. The amount of radioactivity is doubled between 6 and 12 hr, and between 12 and 18 hr there is no further enhancement of incorporation. These results may be interpreted as follows. (a) The DNA polymerase begins to synthesize during the first 6 hr of germination, probably in the late stage of this period. (b) The amount of enzyme synthesized is doubled in the next 6 hr. (c) The synthesis of the enzyme is continued for 12 to 18 hrs of germination.

The de novo and continuous biosynthesis of DNA polymerase may, therefore, be regarded as one of the control mechanisms in the germination of wheat embryos.

**LITERATURE CITED**


