Onset of Deoxyribonucleic Acid Synthesis in Germinating Wheat Embryos

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ABSTRACT

Germinating wheat embryos (Triticum vulgare var. Florence) synthesize proteins before the onset of DNA synthesis. The onset of DNA replication occurs at about 15 hours of germination and was shown to depend on proteins synthesized before 9 hours of germination with the use of blasticidin S, a specific inhibitor of protein synthesis. A 10-fold increase in the activity of DNA-dependent DNA polymerase was found in extracts derived from germinated embryos, as compared to the activity found in extracts from ungerminated embryos.

Water imbibition by the dry seed triggers an array of molecular events which cause the ungerminated seed to change from the quiescent stage to an active growing stage. Marcus et al. (14) showed that protein synthesis is activated in germinating wheat embryos after 30 min of water imbibition. On the other hand, active DNA synthesis cannot be observed in such embryos before 15 hr of germination (3). This sequence of events suggests that the early synthesized proteins may be prerequisites for the subsequent DNA replication.

Kogoma and Lark (11) suggested that protein synthesis is necessary to initiate, but not to sustain, DNA replication in Escherichia coli. Evidence for the presence of structural and initiating proteins was reported by Lark and Lark (12) for DNA synthesis in E. coli. Yoshikawa (20) showed that germinating spores of Bacillus subtilis which have entered the replication cycle can complete the cycle in the presence of chloramphenicol. On the other hand, continuous protein synthesis is required for DNA replication in Chlorella pyrenoidosa (18), and simultaneous synthesis of nuclear proteins is required for DNA synthesis in lily (8). Most of the evidence for the dependence of replication on protein synthesis shown by Jakob and Bovey (9) in Vicia and Harris (7) in mammalian cells was obtained using inhibitors of protein synthesis and the exact nature of the proteins is unknown. The temporal relation between protein synthesis and DNA replication in the germinating wheat embryo offers an opportunity to determine how early synthesized proteins control both the initiation and continuation of DNA synthesis.

In the present work, germinating wheat embryos were exposed to blasticidin S, a specific protein synthesis inhibitor, and the effects of the drug on DNA replication were studied.

MATERIALS AND METHODS

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 (10). Labeled amino acids and thymidine were obtained from the Radiochemical Centre, Amersham, England. Labeled and unlabeled deoxynucleotide triphosphates were obtained from Schwarz BioResearch, Inc. Blasticidin S was a gift of Dr. Noguchi, Kyoto University.

Radioactivity was measured in the Packard Tri-Carb scintillation counter in a standard toluene scintillator.

Germination of the Embryos. The embryos were germinated on 1% (w/v) agar containing 2% (w/v) sucrose in 6-cm Petri dishes. Germination was initiated by imbibition of 500-mg embryos with 1 ml of 2% sucrose solution.

Amino Acid Incorporation. Protein synthesis was measured by the incorporation of radioactive amino acids into acidsoluble material. The embryos were pulsed for 10 or 15 min with 2% sucrose solution containing 14C-amino acids (2 μCi/ml, 52 μCi/milligram). After the pulse the embryos were washed with 2% sucrose solution, homogenized in cold 5% trichloroacetic acid, and heated at 100°C for 15 min to deacylate charged tRNA.

Samples were collected onto GF/C filters. When uptake was measured the filters were directly dried and counted in the scintillator liquid. When incorporation was measured, the filters were washed with 40 ml of cold 5% trichloroacetic acid, 10 ml of alcohol-ether (3:1, v/v), and 10 ml of ether; dried; and counted.

Thymidine Incorporation. DNA synthesis was measured as 3H-thymidine incorporation in acid-precipitable material. The pulse length with 3H-thymidine (2 μCi/ml, 58 μCi/millimole) was 1 hr and the treatment of the embryos was identical with the one used for protein synthesis determination, except that the trichloroacetic acid extract was not heated.

Preparation of Crude Extract. Lots of 500 mg of wheat embryos were homogenized in 10 ml of a buffer consisting of 10 mM tris-HCl, pH 7.6; 20 mM KCl; and 10 mM MgCl2. The homogenate was centrifuged at 10,000 rpm for 30 min in the Sorvall centrifuge. The supernatant was dialyzed against a buffer consisting of 20 mM tris-HCl, pH 7.6; 10 mM KCl; 1 mM MgCl2; 1 mM 2-mercaptoethanol; and 10% (w/v) glycerol. The extract was then centrifuged at 10,000 rpm for 30 min in the Sorvall centrifuge. The supernatant was used as crude extract in the assay for DNA-dependent DNA polymerase. All steps were carried out at 0 to 4°C.

Assay of DNA-dependent DNA Polymerase. The reaction mixture contained, in a total volume of 0.25 ml, 20 mM tris-HCl, pH 7.6; 4.4 mM MgCl2; 16 mM KCl; 0.1 mM each of dATP, dCTP, and dGTP; 1 μM of 1H-TTP (17.4 c/mmole); 4% (w/v) glycerol; 0.4 mM 2-mercaptoethanol; 2 mM ATP; 39 μM of alkali-denatured, ungerminated wheat embryo DNA; and 360 μg of proteins of crude extract from germinated embryos or 630 μg of proteins of crude extract from ungermi-
nated embryos. The incubation was carried out at 35 C. Incorporation was proportional to protein concentration.

The reaction was stopped by adding 1 ml of a salmon sperm DNA solution (66 μg/ml), 0.5 ml of a saturated solution of Na₂HPO₄, 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 onto GF/C filters. The filters were washed with 25 ml of cold 5% trichloroacetic acid, 10 ml of ethanol-ether, and 5 ml of ether; dried; and counted.

![Graph](image1.png)

**FIG. 1.** Effect of blasticidin S concentration on early amino acid incorporation. Lots of 300 mg of wheat embryos were germinated in standard germinating medium containing the indicated amount of blasticidin S for 12 hr. The embryos were washed and transferred into 0.6 ml of germinating medium containing both blasticidin S at the indicated concentration and ³⁵C-amino acid mixture (2 μc/ml, 52 mc/millimole) for 10 min. The embryos were washed with 30 ml of germinating medium, homogenized in cold 5% trichloroacetic acid, and heated to 100 C for 15 min. Samples were collected by filtration on GF/C filters; washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted.

![Graph](image2.png)

**FIG. 2.** Inhibition of ³⁵C-thymidine incorporation by blasticidin S. Embryos (300 mg of embryos per dish) were germinated in a standard germinating medium containing blasticidin S at the concentration of 5 μg/ml. At the indicated times 300 mg of embryos were washed and transferred into 0.6 ml of germinating medium containing blasticidin S and ³⁵C-thymidine (2 μc/ml, 58 mc/millimole) for 1 hr. After the pulse the embryos were washed with 30 ml of germinating medium and homogenized in cold 5% trichloroacetic acid. Samples were collected by filtration on GF/C filters; washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted. Blasticidin S was omitted in the control samples.

![Graph](image3.png)

**FIG. 3.** Beginning of blasticidin S-insensitive period for ³⁵C-thymidine incorporation. For each experimental point 300 mg of embryos were germinated in standard germinating medium. At the indicated times the embryos were transferred into 0.6 ml of germinating medium containing blasticidin S (12.5 μg/ml) and germination was continued until the 18th hr of germination. Embryos were then transferred into 0.6 ml of the medium containing both blasticidin S and ³⁵C-thymidine (2 μc/ml, 58 mc/millimole) for 1 hr. The embryos were washed with 30 ml of germinating medium, homogenized in cold 5% trichloroacetic acid, and collected on GF/C filters. The filters were washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted. Incorporation at 18 hr of germination in the absence of blasticidin S was taken as 100% (65,000 cpm per 300 mg of embryos).

ml of cold 5% trichloroacetic acid, 10 ml of ethanol-ether, and 5 ml of ether; dried; and counted.

Wheat embryo DNA was prepared according to the procedure of Marmur (15) and further purified according to the method of Chen and Osborne (3). Protein concentration was determined according to the method of Lowry et al. (13) with bovine serum albumin as a standard.

**RESULTS**

The Effect of Inhibition of Early Protein Synthesis on the Onset of DNA Synthesis. The dry wheat embryo does not enter the S phase before 8 hr of germination (3) while protein synthesis is activated after 30 min of germination (14). The onset of DNA synthesis may be dependent on the synthesis of certain G₁ proteins which are necessary for the initiation and replication of DNA. Such a possibility can be elucidated by using a specific inhibitor for blocking protein synthesis and analyzing subsequent DNA synthesis. Blasticidin S was reported by Takeuchi et al. (17) to block protein synthesis. Yamaguchi and Tanaka (19) reported that blasticidin S acts by interfering with the elongation of the peptide chains. The effect of this drug on protein synthesis in the germinating wheat embryo was studied in the following experiments.

As presented in Figure 1, complete inhibition of protein synthesis is observed at concentrations as low as 5 μg of blasticidin S per ml of germinating medium. Germination was...
heated medium containing were embryos of medium. At times.

The sensitivity acid. The subject was subjected to incubation was terminated. The pulse was given to 18 hr. Germination was then terminated by homogenizing in cold 5% trichloroacetic acid. The results of such an experiment are in Figure 3. DNA synthesis is complete in the absence of sensitivity due to the addition of 12.5 µg/ml of medium containing blasticidin S (12.5 µg/ml) for the indicated times. The end of each period the embryos were washed in a medium containing both blasticidin S and 3H-amino acid (2 µc/ml, 52 mc/milliatom) for 15 min. The embryos were then incubated for 30 min at 100 C for 15 min. Samples were collected on GF/C filters; washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted. Embryos germinated for 18 hr were pulsed with 3H-amino acids without blasticidin S and processed as above.

**End of Blasticidin S-sensitive Period for Thymidine Incorporation.**

By allowing the embryos to maintain the early protein synthesis for various lengths of time and then blocking protein synthesis with blasticidin S, one can determine the extent of time required to complete the proteins necessary for the embryos to enter the S phase. Germinating embryos were subjected to pulses of blasticidin S starting at different times during germination. At 18 hr, DNA is normally being duplicated, a pulse of 3H-thymidine was given for 1 hr. Germination was then terminated by homogenizing in cold 5% trichloroacetic acid. The results of such an experiment are presented in Figure 3. DNA synthesis is complete in the absence of sensitivity due to the addition of 12.5 µg/ml of medium containing blasticidin S (12.5 µg/ml) for the indicated times. The end of each period the embryos were washed in a medium containing both blasticidin S and 3H-amino acid (2 µc/ml, 52 mc/milliatom) for 15 min. The embryos were then incubated for 30 min at 100 C for 15 min. Samples were collected on GF/C filters; washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted. Embryos germinated for 18 hr were pulsed with 3H-amino acids without blasticidin S and processed as above.

**DISCUSSION**

The ungerminated wheat embryo is a multicellular organism which is metabolically inactive. The suppressed metabolic activity is not entirely the result of the dehydrated state since water imbibition does not trigger a simultaneous activation of protein synthesis is inhibited to the extent of 75% during the 1-hr thymidine pulse employed.

**An in Vitro Study of the Enhancement of 'H-TTP Incorporation by Supernatants Derived from Ungerminated Embryos and Germinated Embryos.** The fact that blocking early protein synthesis in the germinating embryo eliminates the entrance into S phase suggests that those early synthesized proteins play a major role in the mechanism that controls the switching on of cellular DNA replication. One of the activities of such specific proteins could be measured in an in vitro system, by its ability to catalyze the polymerization of deoxyribonucleotide triphosphates, the reaction being dependent on DNA template.

Extracts from ungerminated embryos were compared for their capacity to catalyze the in vitro incorporation of deoxyribonucleotide triphosphates with extracts obtained from embryos that had already entered the S phase. Extraction of embryos was carried out as described in "Materials and Methods." The results of such an experiment are presented in Figure 5 and they show that the extract from germinated embryos has a 10-fold increase in the rate of 'H-TTP incorporation in the presence of 18-hr germinated embryos. A 10-fold increase is also observed when the activity is expressed as incorporation per number of embryos.

![Graph](image-url)

**Fig. 4.** Effect of blasticidin S on amino acid incorporation at 18 hr of germination. For each experimental point 300 mg of embryos were germinated in standard germinating medium. After 18 hr of germination the embryos were transferred into 0.6 ml of medium containing blasticidin S (12.5 µg/ml) for the indicated times. The end of each period the embryos were washed in a medium containing both blasticidin S and 3H-amino acids (2 µc/ml, 52 mc/milliatom) for 15 min. The embryos were then incubated for 30 min at 100 C for 15 min. Samples were collected on GF/C filters; washed with trichloroacetic acid, ethanol-ether, and ether; dried; and counted. Embryos germinated for 18 hr were pulsed with 3H-amino acids without blasticidin S and processed as above.

**Fig. 5.** In vitro 'H-TTP incorporation by crude extracts of ungerminated and germinated embryos. A crude extract was prepared from 500-µg samples of ungerminated and 18-hr germinated embryos, as described in "Materials and Methods." Each reaction mixture contained, in a total volume of 0.25 ml, 20 mM tri-HCl, pH 7.6; 4.4 mM MgCl₂; 16 mM KCl; 0.1 mM each of dATP, dCTP, and dGTP; 1 µc 'H-TTP (17.4 c/microme); 4% (w/v) glycerol; 0.4 mM 2-mercaptoethanol; 2 mM ATP; 39 µg of alkali-denatured, ungerminated wheat embryo DNA; and 360 µg of proteins of crude extract from germinated embryos or 630 µg of proteins of crude extract from ungerminated embryos. The incubation was carried out at 185 C for the indicated times. After incubation the reactions were stopped by addition of 1 ml of a salmon sperm DNA solution (66 µg/ml), 0.5 ml of a saturated solution of Na₂HPO₄, 0.5 ml of 20% (w/v) trichloroacetic acid, and 4 ml of 5% (w/v) trichloroacetic acid. The precipitate was collected onto GF/C filters. The filters were washed, dried, and counted. Prot.: protein.
all metabolic processes. Early biosynthesis of macromolecules such as proteins (14), different RNA species (5), or DNA (3) is sequentially activated.

Translation of early proteins in the germinating embryo is preprogrammed in mRNA which is conserved in the dry embryo (4) and is activated after 30 min of water imbibition. In the experiment described in Figure 1, this early protein synthesis is completely blocked by 5 μg of blasticidin S per ml, followed by inhibition of the onset of DNA synthesis. Our results demonstrate that the primary effect of blasticidin S is the blocking of protein synthesis as shown by Misato (16), while there is no direct effect on DNA synthesis, once initiated.

Inhibition of protein synthesis between 0 and 9 hr of germination results in complete suppression of DNA synthesis at 18 hr of germination. Inhibition of protein synthesis between 9 and 15 hr of germination shows only partial sensitivity of DNA synthesis when measured at 18 hr. The sensitivity of DNA synthesis to the inhibition of protein synthesis rapidly decreases after 15 hr of germination. Such results imply that early proteins translated between 0 and 9 hr of germination are obligatory for the initiation and replication of DNA. The elimination of these proteins would inhibit the entrance of the embryos into the S phase. If the embryos are allowed to proceed in synthesizing protein beyond 9 hr of germination, the rate of DNA synthesized at 18 hr of germination is inversely proportional to the time of blasticidin S application. It is thus possible to assume that during this period the apparatus required for DNA synthesis is being completed.

An ordinary G1 period in wheat was estimated by Avanzi and Deri (1) to last between 1 hr 20 min and 2 hr 15 min in actively dividing cells. Obviously the 15 hr preceding the entrance of germinating embryos into the S phase are not exclusively an active G1 phase. However, the extended time period recorded here for G1 is probably caused by the over-all low rate of metabolism during the early period of germination.

The limiting steps for the entrance into S phase might be: (a) unavailability of an active DNA template (repression at the chromosomal level) (6); (b) shortage of enzymes involved in the metabolism of DNA precursors and the resulting shortage of DNA precursors; (c) shortage of enzymes directly involved in the polymerization of DNA such as DNA-dependent DNA polymerase, ligase, repair enzyme, etc.; (d) presence of inhibitors of one or more of the mentioned enzymes. The present experiments were not designed to answer all these questions. The possibility that DNA polymerase might be a limiting step was further tested in the experiments whereby supernatants from ungerminated and germinated embryos were tested for their capacity to incorporate [3H]-TTP on wheat DNA template. The results of this experiment do indeed suggest that the availability of an active DNA-dependent DNA polymerase might be one of the limiting steps. Further experiments would be required to establish whether a de novo synthesis of the enzyme molecule or the synthesis of an unknown protein factor required for DNA duplication is the G1 event on which the entrance into the S phase is dependent. The feasibility of such a situation would necessitate the further characterization of the polymerizing system which is now under study and the results of this study will be reported elsewhere.

LITERATURE CITED