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CHARACTERIZATION OF THE EARLY SYNTHESIZED DNA IN GERMINATING *TRITICUM AESTIVUM* EMBRYOS

JERZY BUCHOWICZ, ELZBIETA KRASZEWSKA and JOLANTA EBERHARDT*
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; cytoplasmic non-mitochondrial DNA; biosynthesis; isolation; buoyant density; pyrimidine nucleotide clusters.

Abstract—A radioactive DNA preparation was isolated from the post-mitochondrial supernatant fraction of thymidine-[^{14}C] fed wheat embryos. The isolated *s*DNA† preparation was similar to cytoplasmic non-mitochondrial DNA of other eukaryotic cells. The buoyant density and frequency of pyrimidine nucleotide clusters found for the *s*DNA were, essentially, the same as those found for the nuclear DNA. In contrast to DNA that can be leaked from nuclei or other DNA-containing organelles, the *s*DNA is firmly bound to a protein component. At an early germination stage (6–12 hr), the *s*DNA is the only newly-synthesized DNA fraction that can be isolated from the embryo homogenate. Considerable synthesis of nuclear and organellar DNA starts 18 hr after the beginning of germination, just prior to the first maximum of the cell divisions. It is concluded that wheat embryo cells contain cytoplasmic non-mitochondrial DNA and are able to resume its synthesis at an early germination stage, prior to the first post-dormant round of nuclear DNA replication.

INTRODUCTION

It is now well established that DNA synthesis is resumed by germinating seeds only after previous activation of RNA and protein synthesis. The onset of DNA synthesis, as found for cereal seeds by various investigators [1–5], varies from 4 to 24 hr after the beginning of germination. Some amplification and deletion events [6, 7] as well as a preferential synthesis of mitochondrial DNA [8] at early germination stages have been also observed. Our preliminary observations [9] indicated that, in wheat embryos, incorporation of thymidine-[^{14}C] into acid-insoluble, alkali-insensitive material begins after 6 hr but remains at a low level till *ca* 18 hr of germination. Surprisingly, the early synthesized product seemed to be localized in cytosol rather than in nuclei or other DNA-containing organelles. Attempts were therefore made to isolate the cytoplasmic thymidine-[^{14}C]-labelled material in a relatively undegraded form and to characterize the isolated preparation in respect to its chemical nature, buoyant density and molecular size.

The data obtained indicate that the radioactive product may be compared to cytoplasmic non-mitochondrial DNA of animal sources. Indirect evidence indicates also that DNA found in the post-mitochondrial supernatant is not of nuclear origin.

RESULTS

Time course of thymidine-[^{14}C] incorporation into cytoplasmic and nuclear DNA fractions

When pulse-labelled wheat grains are allowed to germinate, a radioactive high molecular weight product appears in the embryos which can be isolated from the embryo homogenate together with the added DNA carrier (Fig. 1). As shown in Fig. 2, the radioactive product first appears 6 hr after germination begins and can be observed as a component of the post-mitochondrial supernatant, not of the nuclear-plus-mitochondrial pellet. Radioactivity of the *s*DNA† fraction rises on further germination, reaching a maximal value at approximately the 15th hour. Radioactive *p*DNA appears considerably later, 12 hr after the beginning of germination, and its amount increases constantly throughout the period investigated, reaching high values just prior to the first maximum of cell divisions. The appearance of

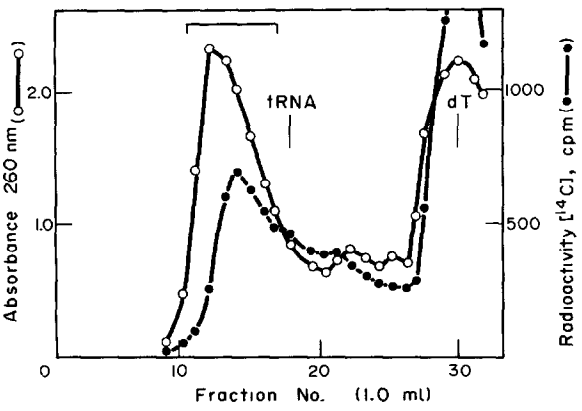

Fig. 1. Sepharose 6B elution profile of RNase and pronase treated post-mitochondrial supernatant fraction from thymidine-[^{14}C]-fed wheat embryos. Embryos from pulse labelled, 15-hr-germinated grains were used. The bracket designates fractions that were pooled for further analyses. Positions of yeast *t*RNA and thymidine as found in a parallel experiment, are indicated.

* Plant Genetics Department, Poznań, Poland.

† Abbreviations used: *p*DNA and *s*DNA designate DNA species found in the 24000 *g* pellet and supernatant fractions, respectively.

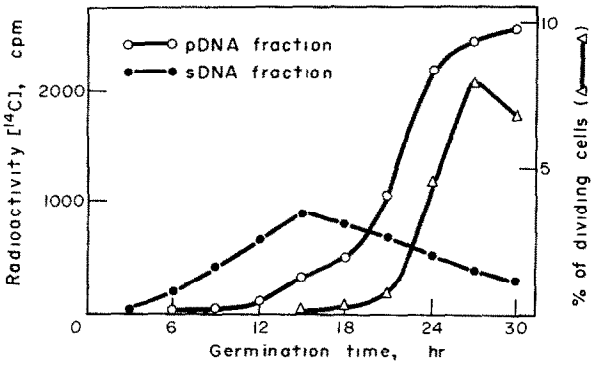

Fig. 2. Time course of thymidine-[^{14}C] incorporation into *s*DNA and *p*DNA fractions in germinating wheat embryos. Pulse labelled wheat grains were germinated for the indicated time periods. Embryos were then separated and used for the isolation of *s*DNA and *p*DNA fractions.

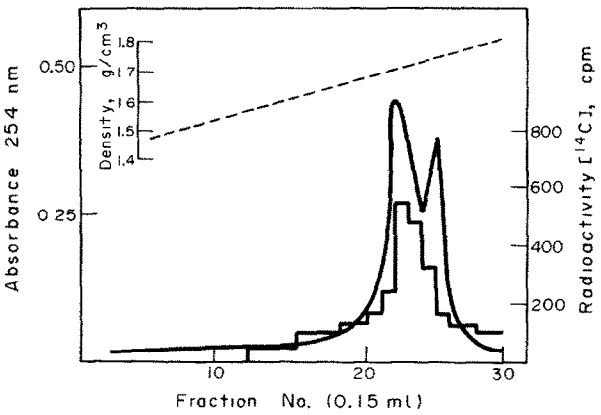

Fig. 3. Distribution in a CsCl density gradient of radioactivity (histogram) and UV-absorbing material (continuous scan) present in the isolated *s*DNA preparation. The preparation (total radioactivity of 4300 cpm) was from 15-hr-labelled embryos. *Micrococcus lysodeikticus* DNA (ρ = 1.731 g/cm^3) was used as marker.

radioactive *s*DNA at an early germination stage, when both nuclear and mitochondrial DNAs still remain completely inactive, and the decrease of *s*DNA radioactivity at later stages, when *p*DNA is highly radioactive, seem to contradict its nuclear or organellar origin.

To check the possibility of artefactual formation of radioactive *s*DNA, a series of controls, as summarized in Table 1, were carried out. It is clear from experiment number 2 that radioactive *s*DNA does not arise from *in vitro* thymidine-[^{14}C] incorporation during embryo homogenization and homogenate fractionation. Similarly, the leakage of radioactive nuclear material is negligible or, at most, moderate (expts 3 and 4). Moreover, the leaked material differs from that obtained as the *s*DNA fraction under the standard conditions. The former can be isolated from the post-mitochondrial supernatant by both I and II DNA preparation methods, the latter only by method I. As these two methods differ in the deproteinization procedure (pronase in the first case and phenol in the second), it may be assumed that the *s*DNA fraction, in contrast to DNA that can be leaked from nuclei, is firmly bound to a protein component and thus cannot be identified with the nuclear leakage products. A prolonged preincubation of the embryo homogenate does result in a considerable contamination of the *s*DNA fraction with the nuclear material. Even in this case, however, the leaked material can be distinguished from typical *s*DNA by its ability to come into the water phase under the phenol method conditions (compare expts 1 and 5, Table 1).

Table 1. Summary of tests for artefactual formation of radioactive *s*DNA fraction

Expt No.	Test conditions	Radioactivity of isolated *s*DNA (cpm) Method I	Method II
1	Standard procedure (see Experimental)	3960	<10
2	Thymidine-[^{14}C] added on homogenization	<10	<10
3	Non-radioactive supernatant mixed with radioactive pellet	110	130
4	Non-radioactive supernatant mixed with radioactive intact nuclei	85	90
5	Non-radioactive supernatant mixed with radioactive pellet and preincubated	340	370

All the subcellular fractions were obtained from 350-embryo samples separated from wheat grains after 18 hr germination either in the presence (radioactive fractions) or in the absence (non-radioactive fractions) of thymidine-[^{14}C]. The supernatant and pellet fractions were separated from each other by centrifugation of the embryo homogenate at 24000 *g* for 20 min. The intact nuclei were prepared according to the method of ref. [26]. The radioactive pellet and intact nuclei contained 2540 and 2180 cpm of ^{14}C radioactivity in the form of DNA, respectively. Preincubation was for 1 hr at 37°. The mixed preparations were homogenized and centrifuged to obtain 24000 *g* supernatant which was used for the isolation of the *s*DNA fraction as described in the Experimental, where differences between the DNA isolation methods I and II are also given.

These observations indicate that there is a stage in the wheat germination process at which *s*DNA synthesis already proceeds but nuclear DNA replication, apparently, still remains untriggered. The nature of the *s*DNA fraction was therefore investigated. Embryos of continuously labelled (15 hr) wheat grains were chosen as the source of the *s*DNA fraction for this investigation.

Sedimentation properties

To get preliminary information on the nature of the *s*DNA fraction, the isolated preparation was centrifuged to equilibrium in a CsCl gradient. The profiles for the radioactive material and accompanying carrier DNA are shown in Fig. 3. The average buoyant density of the radioactive material (*ca* 1.708 g/cm^3) was found to be within the range of ρ values observed for DNA samples. In particular, this buoyant density is close to that of wheat germ nuclear DNA (1.703 g/cm^3) and may correspond to a subcomponent of the main wheat germ DNA band. On the other hand, no radioactivity was found in the *Micrococcus lysodeikticus* DNA zone, even if the foreign DNA was already added at the initial step of *s*DNA extraction. This observation again supports the assumption that radioactive *s*DNA originates from *in vivo* synthesis and does not arise from an artefactual attachment of thymidine-[^{14}C] to the DNA primer. A rather sharp band formed in the CsCl gradient indicates that the radioactive product has a high MW.

Table 2. Distribution of radioactivity among pyrimidine oligonucleotides resulted from acid hydrolysis of *s*DNA preparation.

Nucleotide	% of total pyrimidines	% of total radioactivity	Frequency ratio (radioactive: non-radioactive)
Tp_2	12.7	11.2	0.88
Cp_2	5.0	4.6	0.92
T_2p_3	5.0	4.3	0.86
CTp_3	4.8	4.7	0.98
C_2p_3	3.2	2.2	0.69
CT_2p_4	3.2	3.5	1.09
T_3p_4	3.1	2.8	0.90
C_2Tp_4	2.5	2.1	0.84

The *s*DNA preparation was from 15-hr-labelled embryos. Orotate-[^{14}C] was used as the DNA precursor, and carrier was added as usually. The preparation (1 mg, total radioactivity of 8500 cpm) was hydrolysed (0.1 M H_2SO_4, 100°, 35 min) and the resulted pyrimidine oligonucleotides were separated by 2D PC [30].

in agreement with its behaviour on the Sepharose column (compare Fig. 1).

Chemical data

The isolated *s*DNA preparation could be converted to low-molecular-weight products by either DNase or dilute acid treatments. In the latter case, the resulted degradation products were separated and the predominant pyrimidine oligonucleotides were identified and assayed for radioactivity. When thymidine-[^{14}C] was used as the precursor, radioactivity was found in all thymine-containing oligonucleotides and, at much lower proportions, also in pCp and pCpCp. When, however, orotate-[^{14}C] was used, all the pyrimidine nucleotides were labelled to a similar extent. Table 2 summarizes data on the amounts and radioactivities of the individual pyrimidine oligonucleotides found in the acid hydrolysate of *s*DNA preparation isolated from the orotate-[^{14}C] fed embryos. In general, the frequencies of radioactive oligonucleotides paralleled those of their non-radioactive counterparts derived from carrier. Evidently, the radioactive material underwent conversion to apurinic acid and then hydrolysis to pyrimidine oligonucleotides, just as the carrier did. The resulted degradation products were the same and appeared in similar relative proportions for both radioactive material and carrier (nuclear DNA of wheat germ).

Thus, the isolated radioactive *s*DNA preparation can be unequivocally identified as polydeoxyribonucleotide. Moreover, the *s*DNA fraction seems to be similar to the wheat germ DNA in respect to the frequency of the most abundant pyrimidine clusters. It should be also pointed out that pyrimidine oligonucleotides of the general formula Py_np_{n+1}, as those found in the acid hydrolysate, could originate from either the 5'-end or internal chain positions but not from the 3'-end of the polynucleotide chain [10]. It may be therefore assumed that the radioactive *s*DNA species resulted from *de novo* synthesis rather than from terminal addition of the labelled nucleotide residues to pre-existing polynucleotide chains.

DISCUSSION

Our experiments show that wheat embryos contain a DNA fraction which can be isolated from the tissue homogenate as a component of the post-mitochondrial supernatant. The isolated *s*DNA preparation is similar to microsome-associated DNA species of mammalian cells [11–15]. Simultaneously, it resembles also Okazaki-type fragments, in particular, those isolated by Sakamaki *et al.* [16] from *Vicia faba* embryos.

During the primary stage of cereal seed germination, a series of biosynthetic events is triggered in an ordered, sequential manner [1, 2, 17, 18]. The initiation of *s*DNA synthesis occupies a special position in this sequence of events. Namely, it is resumed at a time when RNA and protein syntheses are already activated but, apparently, prior to the first post-dormant round of nuclear DNA replication. The early appearance of radioactive *s*DNA, at a time when the *p*DNA fraction still remains completely free of the label, makes it highly probable that *s*DNA is synthesized within the cytoplasmic cell compartments and originates neither from the cell nucleus nor from other DNA-containing organelles. Although the occurrence of DNA polymerase-α in the cytoplasm has been recently denied [19, 20], there is a body of evidence in favour of cytoplasmic localization of DNA-synthesizing enzymes in a variety of eukaryotic cells, including wheat embryo cells [21, 22]. Some of DNA polymerases are also able to use RNA as the template [23]. We speculate, therefore, that *s*DNA may be synthesized on those transcripts which reach microsomal structures but are not translated. Such a reverse transcription could be related to the cell differentiation process, in which both cytoplasmic non-mitochondrial DNA [24] and reverse transcriptase [25] may be involved.

EXPERIMENTAL

The grain of wheat (*Triticum aestivum*, cv. Kutnowska) was similar to that used previously [2]. Samples of the grain were surface sterilized with 1% NaOCl and germinated under sterile conditions in a medium containing 10 μg/ml of chloramphenicol, in all expts.

Labelling conditions. Samples of *ca* 350 intact grains were germinated under conditions of pulse or continuous labelling with thymidine-[^{14}C]. For pulse labelling, the sample was poured with 10 ml of a thymidine-[2-^{14}C] soln (2 μCi/ml, 53 Ci/mol) and left for 8 hr at 2°. Then, the sample was rinsed thoroughly with sterile H_2O and allowed to germinate at 22° for a scheduled time (0–30 hr). For continuous labelling, the sample was poured with a similar thymidine-[^{14}C] soln and kept at 22° for 15 or 18 hr, as specified in the Result section. In some auxiliary expts (summarized in Table 2), orotic acid-[6-^{14}C] (5 μCi/ml, 61 Ci/mol) was used instead of thymidine-[^{14}C] for the continuous labelling. In all cases, at the end of germination, embryos were separated manually from the rest of the grain and, in samples of 350 embryos, used for the isolation of radioactive products.

Fractionation of the embryo homogenate. The isolated embryos were ground with a pestle and mortar in ice-cold 0.25 M sucrose containing 10 mM KPi (pH 8.0) and 0.1 mM EDTA. Usually, 5 ml of the soln were used per 350-embryo sample. Nuclear DNA of wheat germ was added (5 mg per sample) as carrier and the homogenate was filtered through a nylon screen (pore size, 35 μm). The filtrate was centrifuged at 24000 *g* for 20 min at 4° and the resulted supernatant and pellet were used for the isolation of *s*DNA and *p*DNA, respectively. Intact nuclei were isolated according to the method of ref. [26].

DNA isolation. Method I was according to ref. [27], including pronase and RNase treatments and Sepharose 6B column chromatography, but with the omission of $CHCl_3$–*n*-BuOH treatment. Method II was according to ref. [28] and its use was

limited to expts quoted in Table 1. Due to the use of carrier, each of the isolated DNA preparations was a mixture of the added non-radioactive DNA and endogenous DNA (expected to be radioactively labelled).

CsCl *buoyant density centrifugation.* Buoyant densities of DNA samples were determined by centrifugation to equilibrium in CsCl gradients. Starting solns (4.5 ml) contained 400 μg of sDNA preparation and sufficient CsCl to give a density of 1.600 g/cm^3 in 0.15 M NaCl, 0.015 M Na citrate, 1 mM EDTA. *Micrococcus lysodeikticus* DNA was used as a density marker and that was added either at initial step of sDNA isolation or immediately prior to the gradient centrifugation. The solutions were centrifuged in a Beckman model L5.75 ultracentrifuge (SW 50.1 rotor) at 36000 rpm for 60 hr at 20°. Fractions of 0.15 ml were collected and absorbance monitored with an ISCO density gradient fractionator. Each fraction was assayed for radioactivity using Bray's [29] scintillator. The density of the CsCl soln of every fifth fraction was determined by using a Zeiss refractometer.

Acid hydrolysis and isolation of pyrimidine oligonucleotides. The preparation of sDNA was converted to apurinic acid and hydrolysed to pyrimidine oligonucleotides under conditions described by Shapiro and Chargaff [30]. The resulted nucleotides were separated by 2D PC [30]. The most abundant of them were identified according to their R_f values, spectral characteristics and phosphorus-to-absorbance ratios. The molar concns and radioactivities of the identified products were then determined. The extinction coefficients were taken from ref. [30].

Frequency of mitoses. Mitotic index data were obtained from Feulgen squashes of root and shoot apex meristems fixed after various periods of germination as described in ref. [31]. Each mitotic index was evaluated by counting cell divisions among 1500 cells. Average data for root and shoot meristems are given.

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