Short Communication

Thymidine-phosphorylating Activity in \( \gamma \)-Plantlets

SEPARATION OF ONSET OF ACTIVITY FROM DEOXYRIBONUCLEIC ACID SYNTHESIS

Received for publication November 27, 1972

OTTO J. SCHWARZ\(^1,2\) and ALAN H. HABER

The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences and Biology Division, Oak Ridge National Laboratory;\(^3\) Oak Ridge, Tennessee 37830 and Department of Botany, The University of Tennessee, Knoxville, Tennessee 37916

Thymidine-phosphorylating capacity, mediated by thymidine kinase or a nucleoside phosphotransferase, is closely correlated with DNA synthesis and cell division in a wide variety of bacterial, animal, and plant systems (4, 5, 11, 14, 17, 22). In cells that are not undergoing DNA synthesis and cell division, these enzymes are usually low or undetectable. Conversely, systems undergoing rapid DNA synthesis and cell division, such as regenerating rat liver and exponentially growing HeLa cells, have high capacities for thymidine phosphorylation (3, 6, 17). Those organisms that lack thymidine-phosphorylating enzymes are unable to incorporate thymidine into their DNA (9). In higher plants, thymidine-phosphorylating enzymes may be involved in pyrimidine nucleotide metabolism during germination and early seedling growth (1, 10, 19, 21).

Our primary purpose in this paper is to test the hypothesis that the control mechanisms for onset and development of thymidine-phosphorylating enzyme activities are separable from DNA synthesis. The results are based upon a study of thymidine phosphorylating enzyme activities in \( \gamma \)-plantlets and unirradiated controls. \( \gamma \)-Plantlets are seedlings growing without DNA synthesis and cell division after \( \gamma \)-irradiation of dry wheat grains (12). Despite high doses received before sowing, they fail to show certain radiation effects that typically result in physiological injury and, apart from the absence of cell division, are therefore remarkably normal (12).

In addition to possible implications for organismal development, the study reported here is relevant to the usefulness of \( \gamma \)-plantlets for two reasons. (a) Since thymidine-phosphorylating capacity is sensitive to general physiological insults, including ionizing irradiation (17), any perturbations in its development would indicate possible injury resulting from the irradiation treatment used to produce \( \gamma \)-plantlets; and (b) a frequently used method of checking for the absence of DNA synthesis in \( \gamma \)-plantlets involves checking for the absence of thymidine incorporation (13) and therefore presupposes the capacity to phosphorylate thymidine.

\(^1\) Postdoctoral Investigator supported by a Research in Aging Training Grant; United States Public Health Service Grant HD 00296 from the National Institute of Child Health and Development. Partial support was provided by National Institutes of Health Biomedical Science Support Grant RR07088-04.

\(^2\) Present address: Department of Botany, University of Tennessee, Knoxville, Tenn. 37916.

\(^3\) Operated by Union Carbide Corporation for the United States Atomic Energy Commission.

MATERIALS AND METHODS

We used wheat, *Triticum vulgare* Vill. (*Triticum aestivum* L.) var. Lemhi. \( \gamma \)-Plantlets were produced by giving 500 krad of \( ^{60} \text{Co} \) \( \gamma \)-rays to dry (10–12% moisture) grain followed immediately by sowing. Irradiation was from a GammaCell 200 unit, from Atomic Energy of Canada, Ltd., Ottawa, at a dose rate of 3.6 krad/min. \( \gamma \)-Plantlets and unirradiated controls were germinated and grown in covered glass dishes containing a water solution of streptomycin (100 \( \mu \)g/ml) under a 16-hr photoperiod of approximately 750 ft-c mixed incandescent and fluorescent white light. Day temperature was 21 ± 1 C, and night temperature was 18.5 ± 1 C.

Samples of 150 embryos were harvested every 12 hr. DNA determinations (7, 20) were performed on two subgroups of 25 embryos per experimental replicate. The two remaining groups of 50 embryos each were used as enzyme-source replicates. The embryos were excised, immediately rinsed in distilled water, treated for 10 min in 1.5% NaOCl, and again rinsed in distilled water. Homogenates were prepared in cold (0–4 C) sodium-potassium phosphate buffer (0.2 M, pH 7.0) by grinding with mortar and pestle and a small amount of quartz sand. The homogenates were filtered through glass wool, centrifuged in the cold at 32,000g for 30 min, and the supernatant was used as the enzyme source. Thymidine phosphorylation was measured by the method of Schwarz and Fites (19). The assay mixture (total volume, 120 \( \mu \)l) consisted of stock solutions of: \( ^{3} \text{H} \)-thymidine (2 \( \times \) 10\(^{-5}\) \( \mu \)M) (0.45 \( \mu \)C/\( \mu \)l), 20 \( \mu \)l; phosphate donor (50 mM) and MgCl\(_{2}\) (4 mM), 50 \( \mu \)l; enzyme preparation, 50 \( \mu \)l; all in sodium-potassium phosphate buffer (pH 7.0), giving a final concentration of 0.2 M. The three phosphate donors studied were AMP, ATP, and phenylphosphate. The protein content did not exceed 2 mg/ml. The reaction mixtures were incubated at 30 C for various times chosen to give not more than 25% phosphorylation of substrate and first-order kinetics (19). The reactions were terminated by the addition of 300 \( \mu \)l of methanol, rapidly cooled to 0 C in an ice bath, and centrifuged to sediment the precipitates. The supernatants were spotted on Whatman No. 3MM paper and chromatographed in water-saturated butanol in the presence of carrier thymidine and thymidine monophosphate. After detection of the nucleosides and nucleotides with short wave ultraviolet radiation, the fractions were cut from the chromatograms and counted in a scintillation counter using PPO-POPOP toluene as the scintillating fluid.

Enzyme activity was determined as the percentage of nu-
nucleoside phosphorylated/min, where 1% phosphorylation equals 0.4 mole thymidine monophosphate produced. In crude enzyme extracts, it is difficult to compare an enzyme activity at different stages of development, because there may be fluctuating activities of competing reactions. In this study, the competing phosphatase activity is of concern in determining absolute enzyme activities for thymidine phosphorylation. Since the levels of phosphatase are approximately constant throughout the first 96 hr of wheat germination (5), any time-dependent increase in capacity for thymidylate production should be a reflection of a true increase in absolute activity of the thymidine-phosphorylating enzymes. The thymidine-phosphorylating enzyme should, nevertheless, be considered as net, rather than absolute, values. All activity values reported represent eight replicate assays, four from each of two groups of 50 seedlings. All experiments were repeated to show reproducibility.

RESULTS AND DISCUSSION

Thymidine-phosphorylating activities and DNA contents of germinating γ-plantlets and unirradiated controls are shown in Figure 1. Twelve hours after sowing, using AMP as a phosphate donor, we found measurable phosphorylation (0.05% phosphorylation/min·50 seedlings). The activity thereafter increases in both γ-plantlets and unirradiated controls. The unirradiated controls show increasing capacity to use AMP as a phosphate donor until 72 hr after sowing, after which an apparent leveling off occurs. In γ-plantlets, phosphorylating activity, using AMP as donor, is still increasing at 96 hr.

Thymidine phosphorylating activity using ATP as the phosphate donor was not measurable under these experimental conditions at 12 hr in either test system. At 24 hr there is measurable activity (0.05% phosphorylation/min·50 seedlings) which subsequently increases. The unirradiated controls again show a leveling off of the phosphorylating capacity at 72 hr.

The lower activity levels in the γ-plantlet presumably reflect a generally reduced synthetic capacity resulting from the absence of mitosis. (The dry weight per γ-plantlet seedling at 96 hr was about half that of the unirradiated controls.)

In the top half of Figure 1, the results with unirradiated wheat agree with the many instances in which thymidine-phosphorylating activity appears or increases approximately when DNA synthesis begins (4, 9, 17, 22). In the lower half of Figure 1, however, the results with γ-plantlets show that the capacity to phosphorylate thymidine can be experimentally uncoupled from DNA synthesis.

Significant thymidine-phosphorylating activity appears in the 12- to 24-hr interval in both the irradiated and control systems. The data in Figure 1 suggest that the γ-irradiation treatment has had little or no effect on the timing of the onset of thymidine-phosphorylating capacity. Up to 36-hr γ-plantlet embryos are difficult to excise from the remainder of the seed. The slightly higher initial DNA values in γ-plantlets (relative either to later times in γ-plantlets or to 12 hr in unirradiated controls) reflect a greater difficulty in removing all residual tissue from the embryos. DNA determinations showed no significant increase in DNA content per γ-plantlet seedling during germination. This is in marked contrast to the approximately 10-fold increase in DNA in control plants during the same experimental period. In γ-plantlet root and shoot apices, autoradiographic studies with 3H-thymidine showed no incorporation into DNA at times (48, 72, and 96 hr), when thymidine-phosphorylating activity was present. Corresponding studies with unirradiated controls did show 3H-thymidine incorporation.

The predominant enzyme activity, both in γ-plantlets and in unirradiated controls, is probably a nucleoside phosphotransferase, because AMP is more effective than ATP as a phosphate donor. It is questionable to what extent, if any, thymidine kinase activity occurs, because ATP may merely serve as a source of AMP for a phosphotransferase reaction (1, 4, 5, 10, 16). An additional line of evidence, from both γ-plantlets and unirradiated controls, further indicating the presence of a nucleoside phosphotransferase is the capacity of phenylphosphate to serve as a phosphate donor.

Using snake venom 5'-nucleotidase, we showed that for each of the three phosphate donors (AMP, ATP, phenylphosphate) all the labeled thymidylate formed was 5'-thymidylic acid in both γ-plantlet and unirradiated control extracts. Also, in both systems we ruled out the possibility that labeled thymidine was degraded to labeled thymine, which reacted with AMP to form adenine and labeled ribothymidine monophosphate; using AMP, there was no incorporation of labeled thymine into a phosphorylated nucleoside. Also, we observed no thymidine phosphorylase activity.

The relatively normal timing and development of thymidine-phosphorylating activity in γ-plantlets indicate that the mechanism controlling this activity can be experimentally uncoupled from DNA synthesis and cell division. This experimental separation is complementary to systems in which DNA synthesis occurs without thymidine-phosphorylating activity (15, 18). The relatively normal developmental profile of thymidine-phosphorylating activity in γ-plantlets is in marked contrast to the perturbations in the profile of this activity after irradiation in certain other systems (2, 8, 14). This contrast may be a further indication of the extent of physiological normality in γ-plantlets apart from inhibition of DNA synthesis and cell division (12). Finally, the presence of thymidine-phosphorylating activity in γ-plantlets helps justify the use of thymidine incorporation to check for the presence or absence of DNA synthesis (13).
Acknowledgments—We thank Larry L. Triplett for assistance in DNA determinations and Rhonda F. Irwin for developing the autoradiograms.

LITERATURE CITED


