Plant Physiol. (1971) 48, 43–45

Enhanced Deoxyribonucleic Acid Polymerase Activity of Chromatin from Soybean Hypocotyls Treated with 2,4-Dichlorophenoxyacetic Acid[1]

Received for publication January 15, 1971

H. R. LEFFLER,[2] T. J. O'BRIEN,[3] D. V. GLOVER, AND J. H. CHERRY
Departments of Agronomy and Horticulture, Purdue University, Lafayette, Indiana 47907

ABSTRACT

Chromatin isolated from soybean (*Glycine max* L., var. Wayne) hypocotyls was capable of catalyzing the polymerization of labeled deoxyribonucleoside triphosphate in the presence of the three other deoxyribonucleoside triphosphates into a trichloroacetic acid-insoluble product. This product was insensitive to base hydrolysis and ribonuclease, but was sensitive to acid hydrolysis and deoxyribonuclease. Chromatin-DNA polymerase required Mg^{2+} and all four deoxyribonucleoside triphosphates for maximal activity. Inorganic pyrophosphate and actinomycin D inhibited the polymerase activity, but 2,4-dichlorophenoxyacetic acid had no effect *in vitro*. Chromatin from plants previously treated with 2,4-dichlorophenoxyacetic acid supported a greater level of DNA synthesis than did chromatin from untreated plants.

Auxin applications were reported by Silberger and Skoog (17) to increase the RNA and DNA contents of cultured tobacco pith cells. Massive proliferation of cells in cucumber hypocotyls treated with 2,4-D was described by West *et al.* (19). Growth, nucleic acid synthesis, and cell division were stimulated by 2,4-D treatments of soybean hypocotyls (9, 10).

Isolated chromatin possesses many characteristics which enable the examination *in vitro* of processes occurring *in vivo* at the time of isolation (1). Chromatin from GA-treated Hazel axes contained more associated RNA polymerase activity than did chromatin from axes of untreated seeds (7). Enhanced levels of chromatin-RNA polymerase activity were found in soybean hypocotyls treated with 2,4-D (14); furthermore, the products of chromatin-directed RNA polymerization differed when soybean hypocotyls were treated with auxin or ethylene (5). Chromatin-RNA polymerase activity of dwarf pea seedlings was enhanced by treatment of the plants with GA (13).

In view of the enhanced levels of chromatin-RNA polymerase activity in 2,4-D-treated soybean hypocotyls and the known increases in DNA content and cell division caused by auxin treatments of hypocotyls, it seemed appropriate to analyze DNA polymerase activity during this "dedifferentiation" process. DNA polymerases have been isolated and characterized from a variety of tissues. Nonbacterial DNA polymerase activity has been isolated from a number of animal sources, including rat liver nuclei (15) and mitochondria (8), sea urchin embryos (12), and cultured human cells (4). Soluble DNA polymerases have been isolated from two plant sources, mung bean (16) and maize seedlings (18). This communication deals with the isolation and characterization of a chromatin-bound DNA polymerase from soybean hypocotyl tissue and the effects of 2,4-D on this activity.

MATERIALS AND METHODS

Soybeans (*Glycine max* L., var. Wayne) were soaked in water overnight and planted in moist vermiculite. They were then grown at 28 C in the dark for 3½ days prior to spraying with 2,4-D. Twenty milliliters of 2,4-D at the appropriate concentration, adjusted to neutrality with KOH, were applied with an atomizer. Hypocotyl sections were harvested at various times after the treatments and kept on ice for no more than 30 min before chromatin isolation.

Chromatin was isolated from 150-g lots of both control and 2,4-D-treated hypocotyls by the method of Huang and Bonner (6). The crude chromatin pellet was suspended in 10 mM tris-HCl, pH 8.0, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol and layered over 20 ml of 2 M sucrose, containing 10 mM tris-HCl, pH 8.0, and 10 mM 2-mercaptoethanol, in a Spinco SW 25.1 centrifuge tube. The upper one-third of the 2 M sucrose was gently stirred with a spatula; the crude gradient was then centrifuged for 3 hr at 20,000 rpm. The purified chromatin pellets obtained after centrifugation were suspended in 3 ml of 10 mM HEPES buffer, pH 7.5. Aliquots of this suspension were used to assay for chromatin-directed DNA synthesis.

The incorporation of labeled deoxyribonucleoside triphosphate into trichloroacetic acid-insoluble material was used to measure DNA polymerase activity. The standard reaction mixture contained, in μmoles, the following: HEPES, pH 7.5, 20; $MgCl_2$, 1; dithiothreitol, 1; dATP,[4] dCTP, and dGTP, 0.05 each; TTP, 0.002; and 5 μc of ^{3}H-TTP at a specific activity of 12 mc/μmole; and chromatin equivalent to 4 to 8 μg of DNA: in a final volume

[1] Purdue University Agriculture Experiment Station Journal Paper 4304. Taken in part from a dissertation submitted by the senior author to the Graduate School of Purdue University in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: Department of Agronomy, University of Illinois, Urbana, Ill. 61801.

[3] Present address: Allan Hancock Foundation, Department of Biological Sciences, University of Southern California, Los Angeles, Calif. 90007.

[4] Abbreviations: dATP: 2′-deoxyadenosine 5′-triphosphate; dCTP: 2′-deoxycytidine 5′-triphosphate; dGTP: 2′-deoxyguanosine 5′-triphosphate; TTP: 2′-deoxythymidine 5′-triphosphate; TMP: 2′-deoxythymidine 5′-monophosphate.

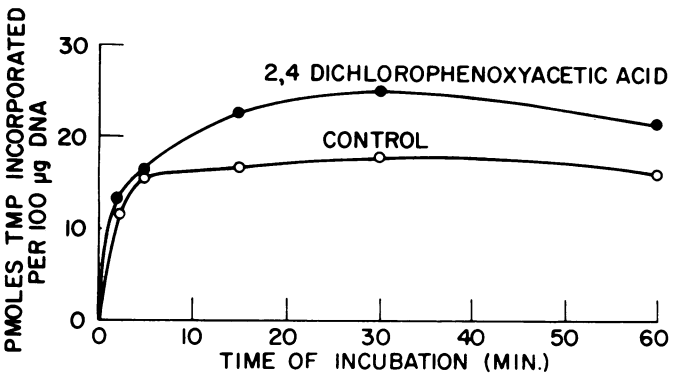

FIG. 1. Time course of TMP incorporation into acid-insoluble product by chromatin from 3-day control and 2,4-D-treated seedlings. The seedlings were sprayed with 100 µg/ml of 2,4-D 18 hr prior to chromatin isolation.

Table I. *Effect of Changes in the Reaction Mixture on Chromatin-DNA Polymerase Activity*

Reaction Mixture	Chromatin-DNA Polymerase Activity
	%
Complete	100[1]
-Mg^{2+}	2
-dATP	29
-dCTP	55
-dGTP	19
-dATP, -dCTP, -dGTP	0
+ Pyrophosphate (1 µmole)	4
+ Actinomycin D (1 µg)	79
+ Actinomycin D (20 µg)	49
+ Actinomycin D (50 µg)	30
+ 2,4-D (10^{-8} M)	99

[1] Data are pooled from several experiments in which the activity observed in the normal reaction mixture was about 40 pmoles of TMP incorporated per 100 µg of DNA.

of 0.2 ml. The reaction mixture was incubated for 30 min at 37 C and stopped by the addition of 3 ml of cold 10% trichloroacetic acid containing 1 mM PPi.

Precipitated material was transferred onto glass fiber filter disks (Whatman GF/A), washed five times with 5-ml portions of cold 5% trichloroacetic acid and dried. The disks were placed in scintillation vials and counted in a Tri-Carb liquid scintillation spectrometer with a counting efficiency of 40% for tritium. Chromatin DNA content was measured by the diphenylamine procedure of Burton (2) after initial hydrolysis in 0.5 N perchloric acid for 45 min at 70 C.

RESULTS

The optimal Mg^{2+} concentration for chromatin-DNA polymerase activity was found to be 5 mM. Observed chromatin-DNA polymerase activity was proportional to the amount of chromatin added to the reaction mixture, within the range of 2 to 8 µg DNA. The incorporation of TMP is seen in Figure 1 to be maximal after 30 min of incubation.

Omission of Mg^{2+} from the reaction mixture decreased chromatin-DNA polymerase activity by 98% (Table I). The absence of dATP or dGTP decreased the activity 70 and 80% respectively, while the omission of dCTP reduced the activity by about 45%. No activity was observed in the absence of these three deoxyribonucleoside triphosphates. Addition of 1 µmole of pyrophosphate, an end product of the reaction, to the assay mixture blocked TMP incorporation by 96%; maximum inhibition of chromatin-DNA polymerase activity with actinomycin D was 70%. No effect of 2,4-D was observed *in vitro*. Chromatin-DNA polymerase activity was reduced only slightly by preincubation treatment with RNase at 2 C; however, TMP incorporation was completely destroyed by DNase (Table II).

Chromatin-DNA polymerase activity was considerably increased by prior treatment of the seedlings with 2,4-D (Table III). There was a 12 hr lag between auxin application and en-

Table II. *Effect of Nuclease Addition to the Reaction Mixture on Chromatin-DNA Polymerase Activity*

Ribonuclease was added to the complete reaction mixture and incubated at 2 C for the indicated periods of time prior to the assay incubation period of 30 min at 37 C. Deoxyribonuclease was added at the initiation of the reaction and allowed to continue for the indicated periods of time beyond the normal 30 min, 37 C incubation. Incorporation in the absence of nuclease was 8000 cpm.

Nuclease Present	Chromatin-DNA Polymerase Activity
	%
None	100
Ribonuclease (50 µg, 10 min)	93
Ribonuclease (50 µg, 20 min)	86
Deoxyribonuclease (50 µg, 5 min)	5
Deoxyribonuclease (50 µg, 15 min)	0
Deoxyribonuclease (50 µg, 30 min)	0

Table III. *Induction of Chromatin-DNA Polymerase Activity by 2,4-D Treatments*

Hours after Treatment[1]	Chromatin-DNA Polymerase Activity
	%
0	100[2]
6	102
12	130
18	211
24	146

[1] Plants were sprayed with 100 µg/ml 2,4-D at the indicated times prior to harvest.

[2] Chromatin-DNA polymerase activity by the control system was 19 pmoles of TMP incorporated per 100 µg of DNA.

Table IV. *Effect of the Concentration of 2,4-D in the Treatments on Chromatin-DNA Polymerase Activity*

Treatment[1]	Chromatin-DNA Polymerase Activity
µg 2,4-D/ml	%
0	100[2]
50	106
250	143
500	246
1000	247

[1] Plants were sprayed with the indicated concentration of 2,4-D 18 hr prior to harvest.

[2] 100% represents the incorporation of 72 pmoles of TMP per 100 µg of DNA.

hancement of chromatin-DNA polymerase activity; maximal enhancement of the enzyme activity required 18 hr. DNA polymerase activity of chromatin from hypocotyls increased as a function of 2,4-D concentration from 50 to 500 μg/ml (Table IV). The highest concentrations of 2,4-D (0.5 and 1.0 mg/ml) gave the same increase.

DISCUSSION

Chromatin isolated from soybean hypocotyls contained an active DNA polymerase. Maximal activity of this enzyme required the presence of all four deoxyribonucleoside triphosphates and Mg^{2+} in the assay mixture. The optimal Mg^{2+} concentration for soybean hypocotyl chromatin-DNA polymerase was found to be 5 mM. This is about half that reported for soluble DNA polymerases isolated from rat liver mitochondria (8) and sea urchin embryos (12), but about the same as the Mg^{2+} concentrations used in the mung bean and maize seedling soluble DNA polymerase assays (16, 18).

Formation of acid-insoluble product by chromatin-DNA polymerase was unaffected by RNase, but was completely destroyed by DNase. This product has also been found to be resistant to alkaline hydrolysis, but susceptible to perchloric acid digestion (11). These results suggest that the product is a DNA-like polymer.

Lack of complete dependence of this chromatin-DNA polymerase activity upon the presence of all four deoxyribonucleoside triphosphates indicated that the activity of the preparation was not that of a pure DNA polymerase. This observation was not expected in that the chromatin-RNA polymerase system of O'Brien *et al.* (14) did demonstrate complete dependence upon the presence of added ribonucleoside triphosphates in the assay mixture. The activity observed in the absence of any single deoxyribonucleoside triphosphate is not due to the formation of a homopolymer of thymidine residues (O'Brien and Leffler, unpublished results).

It has previously been established that treatment of sensitive tissues, such as soybean seedlings, with 2,4-D results in massive accumulations of DNA and RNA associated with the induction of cell division (9, 19). Friedman (3) found that while total DNA polymerase activity of cultured HeLa cells fluctuated little with the stage of the cell cycle, the activity associated with nuclei did increase as the cells approached the S phase and began replication. One might therefore expect an enhancement of chromatin-DNA polymerase activity during the initiation of cell division. Induced mitosis in soybean hypocotyl tissue was first observed about 12 to 14 hr after treatment with 100 μg/ml or higher concentrations of 2,4-D (9). In agreement with this, a 30% increase in chromatin-DNA polymerase activity was observed 12 hr after 2,4-D treatment (Table IV). This increase in chromatin-DNA polymerase activity followed a lag period of 6 hr after 2,4-D treatment during which time the chromatin-DNA polymerase activity remained unaffected. However, O'Brien *et al.* (14) have found substantial increases in chromatin-RNA polymerase activity during this lag period (46% over control 4 hr after treatment with 2,4-D). It is therefore apparent that 2,4-D, at concentrations which induce cell division in soybean hypocotyl tissue, enhances RNA polymerase activity prior to activation of DNA polymerase activity and cell division.

The specific mode of action by which 2,4-D, as well as other auxins, affect nucleic acid metabolism has escaped elucidation. The confusion centers primarily upon the fact that low concentrations of 2,4-D (less than 100 μg/ml) stimulate RNA synthesis, but enhance neither DNA synthesis nor cell division. As was noted in Table IV, there was no increase in chromatin-DNA polymerase activity following treatment of soybean seedlings with 50 μg/ml of 2,4-D. However, after a similar 2,4-D treatment, chromatin-RNA polymerase activity was increased over 100% (14). Furthermore, treatments with 2,4-D at concentrations greater than 100 μg/ml result in enhancement of both chromatin-DNA polymerase and chromatin-RNA polymerase activities. Thus, observations of *in vivo* responses to auxin (RNA synthesis, DNA synthesis, and cell division) are reflected in the respective enzyme activities associated with chromatin isolated from treated tissues.

The differences in the responses of RNA polymerase and DNA polymerase to 2,4-D, and the DNA polymerase assay described in this paper, may be of further use in the elucidation of the mechanism of the herbicidal action of 2,4-D.

Acknowledgment—The assistance given the authors by Mr. J. W. Hardin and Mr. Jan Kanabus was greatly appreciated. This research was supported by an institutional grant to T. J. O. B. and H. R. L. from the American Cancer Society and the Indiana Elks, a Purdue Research Foundation David Ross Fellowship to H. R. L., and by a National Science Foundation grant (GB-4593) to J. H. C.

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