

PERIODIC INCREASE IN DEOXYRIBONUCLEASE ACTIVITY DURING THE CELL CYCLE IN SYNCHRONIZED *EUGLENA*

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INTRODUCTION

The deoxyribonucleases have numerous biological functions in cellular growth and development (1-3). These include repair mechanisms for damaged DNA molecules, protective mechanisms against viral infection, promotion by viral DNases of intracellular viral growth, and intracellular digestive functions carried out by acid nucleases located in the lysosomes. Accumulating evidence indicates that another possible function of DNases is in the control of DNA synthesis. Stern (4) has found a sharp periodicity of DNase activity in lily anthers followed by the appearance of a pool of deoxyribosides. *Escherichia coli* mutants deficient in endonuclease I failed to utilize deoxynucleoside triphosphates for DNA synthesis (5); and nuclease activity has been found associated with, or necessary for, DNA polymerase activity (6-8). Indeed, both nuclease and polymerase activities have recently been shown to be present on the same molecules, suggesting a subunit structure (9).

Although nuclease activity has been shown in bacteria, higher plants, and animal tissues, relatively little work has been done with algae (10). Since *Euglena* can be synchronized by appropriate light/dark cycles and has been shown to synthesize its DNA at a specific time during the cell cycle (11, 12), this organism seemed a useful tool for studying the activity of DNases and their temporal association with DNA synthesis. This note reports that DNA synthesis in *Euglena* occurs in a discontinuous manner beginning approximately 6-7 hr after the onset of the light period, while DNase activity increases approximately at the time of DNA synthesis. Column chromatography indicates that only one active species of DNase exists, having a pH optimum of 7.5. This temporal relation between DNA synthesis and DNase activity suggests a possible control mechanism by the latter and is discussed in relation to evidence in other organisms.

MATERIALS AND METHODS

Cultures of *Euglena gracilis* Klebs (strain Z) were grown synchronously on a modified (13) Cramer and

Myers's (14) minimal salt medium. The experimental cultures (20L capacity) were maintained in environmental chambers at $25^{\circ} \pm 0.5^{\circ}\text{C}$, the doors of which were provided with a bank of six 40w, cool-white, fluorescent bulbs. The cells were synchronized by a light/dark cycle of 10 hr of light and 14 hr of dark (LD: 10, 14) controlled by a clock timer. The cultures were automatically sampled every 2 hr by a miniaturized fraction collector (13) and counted with a Coulter electronic particle counter for monitoring population growth and determining the degree of synchrony. Cells were allowed to reach a concentration of about 20,000 cells/ml. Beginning with the onset of the light period (considered as hour 0 of the cell cycle), four 500-ml aliquots were taken from the cultures at intervals of approximately 2 hr over a 20-hr time-span, centrifuged at 10,400 rpm, and fast-frozen in dry ice-acetone and stored overnight at -18°C for future assays. Two sets of aliquots were used for DNA determinations, and another two sets for enzyme assays.

The frozen pellets used for DNA determinations were extracted four times with cold acetone, once with cold 0.3 N PCA, and twice with 0.5 N PCA at 70°C for 20 min each. The two 0.5 N PCA extracts were combined, and the Burton (15) modification of the diphenylamine colorimetric test for deoxyribose (16) was used for assaying total cellular DNA. Cytoplasmic DNA is only about 3% of the total cellular DNA; therefore, the extraction procedure reflects mainly the synthesis of the nuclear fraction.

The frozen cells used for enzyme assays were suspended in 0.02 M Tris-HCl buffer (pH 7.5) and sonicated for 4 min in an M.S.E. Ultrasonic Disintegrator (60w) at 1.3 amps (Instrumentation Associates, Inc., New York, N. Y.). The homogeneous green suspension was centrifuged for 30 min at 17,000 rpm at 5°C . The supernatants were used directly for enzyme assays.

A radioactive assay was used for determining the activity of DNase(s). *Escherichia coli* B DNA was labeled with thymidine- ^3H (New England Nuclear Corp., Boston, Mass.) and isolated according to the combined procedures of Kirby (17) and Marmur

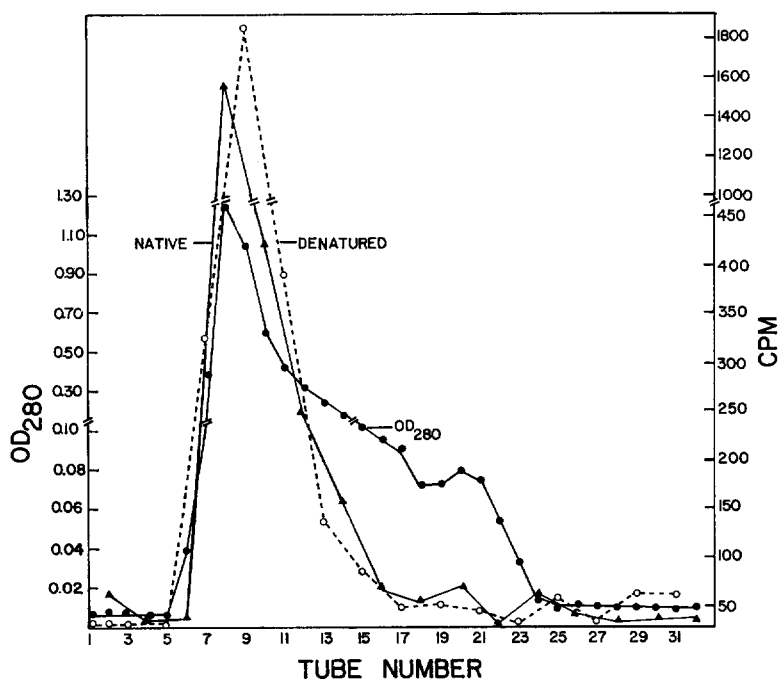


FIGURE 1 Elution profile of *Euglena* DNase after column chromatography on Sephadex G-100. 2 ml of a supernatant fraction from a 40,000 rpm ultracentrifugation of sonicated *Euglena* were eluted with 100 ml of Tris-HCl buffer (pH 7.5) containing 0.05 M NaCl. 2-ml fractions were collected, and protein content (OD_{280}) was assayed on all tubes, while alternate tubes were analyzed for activity with either native or denatured DNA. Enzyme activity was run for 3 hr at 37°C.

(18). Both single- and double-stranded DNA were used as substrates. Single-stranded DNA was obtained by boiling and rapidly cooling the *E. coli* native DNA. The concentration of the stock solution of DNA- 3H was 56.4 $\mu\text{g}/\text{ml}$, and the activity was 7341 cpm/ μg DNA.

The reaction mixture consisted of 0.1 ml of DNA- 3H and 0.1 ml of enzyme extract run at 30°C for 30 min in a gyratory shaker, the reaction being linear for at least 60 min. The reaction was stopped by adding 0.1 ml of thymus carrier DNA (300 $\mu\text{g}/\text{ml}$) and 0.1 ml of 1.0 N PCA. The mixture was centrifuged, and the supernatant was added to 5 ml of Bray's solution and counted in a Packard Tri-Carb Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Illinois).

Sephadex G-100 columns (1.5 \times 25 cm) were run so as to separate the possible DNases existing in supernatant fractions of whole-cell sonicates. Approximately 8 ml of packed cells were suspended in 100 ml of 0.02 M Tris buffer, sonicated, centrifuged at 40,000 rpm for 90 min, and an aliquot of the supernatant was applied to the Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) columns.

RESULTS AND DISCUSSION

Fig. 1 shows the profile obtained from a Sephadex G-100 column. Both native and denatured DNA were used as substrates for determining whether there were several different enzymes, one specific for denatured DNA and another specific for native DNA. From the profile it appears that if, indeed, there are different enzymes, they elute together.

A pH assay was made on the eluant in tubes 8 and 9 from the Sephadex G-100 column, with both single-stranded and double-stranded DNA as substrates. The assay mixture consisted of 0.1 ml of DNA- 3H , 0.1 ml of enzyme extract, and 0.1 ml of buffer (0.1 M) at various pH's. Buffer systems were as follows: Tris-maleate, pH 5-8; Tris-HCl, 7.2-8.6; carbonate-bicarbonate, 9.5-10.5. The mixture was incubated for 3 hr at 37°C and stopped by adding 0.1 ml of thymus carrier DNA and 0.1 ml of 1.0 N PCA. The results of the pH assay indicated that the pH optimum was about 7.5, while another broad peak was found near pH 6.0. Single-stranded DNA as substrate always yielded higher counts than double-stranded DNA.

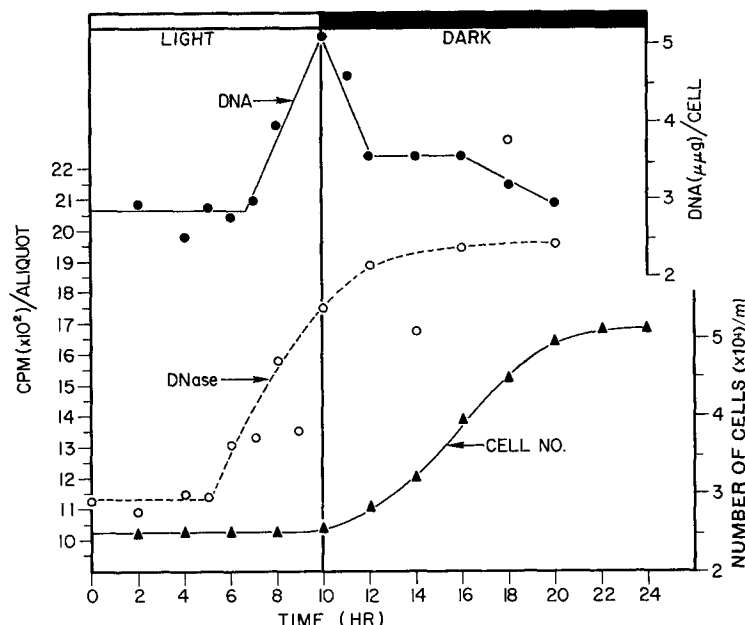


FIGURE 2 Periodic activity of *Euglena* DNase (single-stranded DNA substrate) and discontinuous synthesis of DNA. DNA synthesis (solid circles) begins about 6-7 hr after the beginning of the light period. DNase activity per aliquot (open circles) approximately parallels DNA synthesis. Cell concentration (triangles) was determined at 2-hr intervals and indicates that the culture had been synchronized by the imposed 10:14 light/dark cycle.

It is interesting to note that Norton and Roth (10) observed a maximum activity of DNase from *Anacystis nidulans* at pH 7.0, although the activity of the enzyme(s) was also high at pH's ranging from pH 4.5 to 7.5.

The supernatants from whole-cell sonicates of *Euglena* were also placed on diethylaminoethyl (DEAE) and carboxymethyl cellulose columns and eluted with a continuous gradient of NaCl (0-0.1 M). The eluants were tested for activity, and only one major peak was observed at pH 7.5.

In order to determine whether any activity remained in the residual pellet from the high-speed (40,000 rpm) centrifugation, the pellet was extracted with 0.2% sodium deoxycholate and tested for activity. The activity found was negligible in comparison with that in the supernatant. The supernatant was also treated in the same manner to determine whether the treatment inactivated the nuclease(s). The activity was decreased only slightly. It appears, therefore, that any activity remaining in the pellet was minimal.

Fig. 2 illustrates the activity of DNase (pH 7.5) during the cell cycle of *Euglena*, with single-

stranded DNA as substrate; the pattern shown is representative of a series of such runs. The activity remains at a reduced level until DNA synthesis commences approximately 6-7 hr after the onset of light, at which time the activity of the enzyme also rapidly increases. (When double-stranded native DNA was used as substrate, DNase activity remained at a constant level throughout the entire cell cycle.) The temporal association of DNase activity with DNA synthesis suggests a possible mechanism of control by this enzyme. As previously mentioned, nuclease activity may be necessary to initiate the activity of DNA polymerase or may be a subunit of the polymerase molecule.

In that DNase shows a discontinuous increase of activity during the light in synchronous *Euglena*, this enzyme is unlike many other enzymes studied in our laboratory (e.g., ribulose 1,5-diphosphate carboxylase, TPN-dependent triose phosphate dehydrogenase, alanine dehydrogenase, and acid phosphatase), the activities of which increase linearly throughout the light period in parallel with both total and soluble protein.

Although DNase may be a controlling factor in

DNA synthesis, the possible influence of the other enzymes in the biosynthetic pathway of DNA may also regulate its synthesis. Studies on synchronous populations of *Chlorella pyrenoidosa* have suggested that the control of DNA synthesis in this organism may lie in the enzymes (dCMP¹ deaminase and dTMP kinase) of the dTTP biosynthetic pathway (19, 20). (On the other hand, the recent results of Wanka and Poels do not support the hypothesis that DNA synthesis in *Chlorella* is regulated by thymidylate kinase [21].) Also, preliminary studies by Cook (22) on dTMP kinase and dGMP kinase in *Euglena* indicate that their activities occur early in the light period; thus, the initiation of DNA synthesis in synchronized *Euglena* may stem from earlier events in the division cycle.

Moreover, the data presented here on DNase activity, coupled with those findings of Cook (22) on the activities of dTMP and dGMP kinases, suggest that controls in *Euglena* may occur at different levels. The synthesis of DNA requires the presence of triphosphorylated deoxynucleotides as well as a template. If the phosphorylations occur early in the cell cycle, then perhaps the final control lies in the availability of a template and initiation of DNA polymerase activity. No published data have yet appeared on polymerase activity in the cell cycle of *Euglena*; however, the time of its synthesis and initiation of its activity by DNase would prove most interesting.

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Note added in proof: A recent report on nucleases in *Euglena* by E. F. Carell, J. M. Egan, and E. A. Pratt, entitled "Studies on Chloroplast Development and Replication in *Euglena*. II. Identification of Two Different Deoxyribonucleases", appeared in *Arch. Biochem. Biophys.* 1970. 138: 26.

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¹Abbreviations: dCMP = deoxycytidine monophosphate; dTMP = deoxythymidine monophosphate; dTTP = deoxythymidine triphosphate; dGMP = deoxyguanosine monophosphate.

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