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In Vivo Levels of Diadenosine Tetraphosphate and Adenosine Tetraphospho-Guanosine in *Physarum polycephalum* during the Cell Cycle and Oxidative Stress

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Cellular levels of diadenosine tetraphosphate (Ap_4A) and adenosine tetraphospho-guanosine (Ap_4G) were specifically measured during the cell cycle of *Physarum polycephalum* by a high-pressure liquid chromatographic method. Ap_4A was also measured indirectly by a coupled phosphodiesterase-luciferase assay. No cell cycle-specific changes in either Ap_4A or Ap_4G were detected in experiments involving different methods of assay, different strains of *P. polycephalum*, or different methods of fixation of macroplasmodia. Our results on Ap_4A are in contrast with those reported previously (C. Weinmann-Dorsch, G. Pierron, R. Wick, H. Sauer, and F. Grummt, Exp. Cell Res. 155:171–177, 1984). Weinmann-Dorsch et al. reported an 8- to 30-fold increase in Ap_4A in early S phase in *P. polycephalum*, as measured by the phosphodiesterase-luciferase assay. We also measured levels of Ap_4A, Ap_4G, and ATP in macroplasmodia treated with 0.1 mM dinitrophenol. Ap_4A and Ap_4G transiently increased three- to sevenfold after 1 h and then decreased concomitantly with an 80% decrease in the level of ATP after 2 h in the presence of dinitrophenol. These results do not support the hypothesis that Ap_4A is a positive pleiotypic activator that modulates DNA replication, but they are consistent with the hypothesis proposed for procaryotes that Ap_4A and Ap_4G are signal nucleotides or alarmones of oxidative stress (B. R. Bochner, P. C. Lee, S. W. Wilson, C. W. Cutler, and B. N. Ames, Cell 37:225–232, 1984).

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) and other adenosine tetraphosphonucleoside (Ap_4N) and adenosine triphosphonucleoside (Ap_3N) compounds are synthesized as side reaction products by tRNA synthetases from several organisms, including *Physarum polycephalum* (6, 19, 36, 40, 55). Zamecnik and Stephenson (54) have suggested that Ap_4A might serve as a regulatory nucleotide that couples protein synthesis and transcription or replication. Support for this hypothesis came from the finding that Ap_4A levels vary widely, with high levels correlating with rapid growth and transformation (42). Stronger support came from a report that Ap_4A could induce DNA replication in permeabilized, quiescent cultured cells (21). Ap_4A subsequently was shown to bind to a subunit of DNA polymerase α from thymus (22, 41) and HeLa cells (3), although no effect on the activity of the polymerase was detectable (27, 35). A possible mode of action for Ap_4A was suggested by the observation that it can serve as a primer for DNA polymerase α, utilizing poly(dT) (43) or a double-stranded octadecamer segment of the simian virus 40 replication origin (53) as template. Ap_4A also has been reported to stimulate replication on either endogenous or exogenous templates when injected into *Xenopus* oocytes (57). Zamecnik has reviewed the results that support this hypothesis (52).

In contrast, Ames and co-workers have found in bacteria that levels of Ap_4A and other dinucleoside polyphosphates increase up to 100-fold under a variety of conditions. Aromatic oxidants, sulfhydryl reagents, ethanol, heat shock, and substances that decrease glutathione specifically induce these dinucleotides in nonproliferating *Escherichia coli* and *Salmonella typhimurium* (7, 28, 29). Bochner et al. (7) have proposed that oxidative damage may be a common element in all of these treatments and that Ap_4A and related dinucleoside polyphosphates are signal nucleotides or alarmones that initiate an adaptive response to oxidative stress. Increases of Ap_4A induced by heat-shock and ethanol also have been observed in mammalian cells (J. C. Baker and M. K. Jacobson, Fed. Proc. 43:1728, 1984).

Until recently there were few data available on the changes in in vivo Ap_4A levels during the cell cycle or changes in growth conditions. Studies by Rapaport and Zamecnik (42) mentioned above were done on asynchronous cultures. Plesner et al. (38) have reported an increase in Ap_4A during the early part of the *E. coli* growth curve, but they also found a large increase in Ap_4A after *Tetrahymena pyriformis* was shifted to a starvation salts medium (37). Probst et al. (39) have found that after a shift to anaerobic conditions, the Ap_4A level in Ehrlich ascites cells dropped 100-fold, the adenylate energy charge remained constant, and initiation of replication in new replicons ceased. McLennan and Prescott (31) studied embryos of the brine shrimp *Artemia salina* and found that Ap_4A levels increased 125-fold on activation of dormant embryos, and the peak level corresponded to the onset of DNA replication.

To provide data on Ap_4A changes in a well-defined cell cycle system, we measured Ap_4A and adenosine tetraphospho-guanosine (Ap_4G) in the slime mold *P. polycephalum* using a specific high-pressure liquid chromatographic (HPLC) assay (17). This organism has a naturally synchronous cell cycle which has been studied extensively (47). We found no cell cycle-specific changes in either compound. We also measured Ap_4A and Ap_4G in macroplasmodia subjected to oxidative stress by treatment with dinitrophenol (DNP). The dinucleotides transiently increased by a factor of 3 to 7 after treatment with DNP. Some of these results have been reported previously in abstracts (L. D. Barnes, P. N. Garrison, and S. A. Mathis,

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Fed. Proc. 43:1473, 1984; Hoppe-Seyler's Z. Physiol. Chem. 365:608, 1984).

MATERIALS AND METHODS

Materials. Ap_4A was purchased from Sigma Chemical Co. (St. Louis, Mo.) and custom labeled with tritium (Amersham Corp., Arlington Heights, Ill.). [^{3}H]Ap_4A was purified by chromatography on dihydroxyboronyl-BioRex 70 resin (4) and analyzed for purity by isocratic HPLC (17). Partisil 10 SAX columns and resin for HPLC were purchased from Whatman, Inc. (Clifton, N.J.). Snake venom phosphodiesterase I was obtained from Worthington Diagnostics (Freehold, N.J.). ATP, DNP, tri-*n*-octylamine, D-luciferin, luciferase (chromatographically prepared and crystallized), and *E. coli* alkaline phosphatase (Sigma type III) were purchased from Sigma. The alkaline phosphatase had no detectable phosphodiesterase I activity when assayed with Ap_4A as a substrate using the isocratic HPLC system described previously (17). Matheson Scientific, Inc. (Elk Grove Village, Ill.) supplied 1,1,2-trichlorotrifluoroethane (Freon 113). [*methyl*-^{3}H]thymidine (5 Ci/mmol) was purchased from Amersham.

Culture and treatment of *P. polycephalum*. *P. polycephalum* M_3CVII and M_3CVIII were maintained in shake culture at 25 to 26°C in semidefined medium (12) as described previously (17). Exponentially growing microplasmodia (2 to 3 g [wet weight]/100 ml of medium) were harvested by centrifugation at 250 × *g* for 10 s and suspended in an equal volume of salts medium (20). Portions (1 ml) were spread on filter paper (no. 576; Schleicher & Schuell, Inc., Keene, N.H.) supported on stainless steel mesh in a 15-cm-diameter petri dish to form macroplasmodia by fusion (24). Growth medium (37 ml) was added after incubation for 45 to 75 min. All manipulations with live plasmodia were done in dim red light.

For studies on the cell cycle, macroplasmodia were collected at intervals prior to the second mitosis, and collections were continued through the third mitosis postfusion. For studies on oxidative stress, some macroplasmodia were shifted onto salts medium 30 to 50 min after the second mitosis. DNP was added to a final concentration of 0.1 mM to the medium of some macroplasmodia 8 to 9 h after the second mitosis. Macroplasmodia were collected at intervals from 8 to 13 h after the second mitosis.

Mitotic stages were determined by phase-contrast microscopy on smear preparations fixed in glycerol-ethanol (1:1 [vol/vol]). When DNA replication was to be monitored, 10 μCi of [*methyl*-^{3}H]thymidine was added to each dish 25 to 30 min before collection.

Preparation of acid-soluble extracts. Macroplasmodia were collected by six different methods in an attempt to achieve the most rapid fixation. Method A. Plasmodia were frozen on the filter paper in liquid N_2-cooled isopentane and stored at −80°C. They were later immersed in and scraped into cold 5% (wt/vol) trichloroacetic acid (TCA) and sonicated. Method B. Plasmodia were scraped into liquid N_2-cooled isopentane and later sonicated in cold 5% TCA. Method C. Plasmodia were scraped directly into cold 5% TCA and immediately sonicated. Method D. Plasmodia were frozen on filter paper in liquid N_2-cooled isopentane and lyophilized. The powder was dissolved in cold 5% TCA and sonicated. Method E. Plasmodia were immersed in cold 5% TCA, scraped, and sonicated. Method F. Plasmodia were scraped into a rough line on filter paper, vacuum aspirated into rapidly stirring, cold 5% TCA, and sonicated. In all cases the TCA contained 0.5 to 1.0 pmol of [^{3}H]Ap_4A (2.5 Ci/mmol) to monitor recovery for each plasmodium. A total of 5 ml of 5% TCA was used per plasmodium for all methods, except for those involving immersion, in which 14 ml was used. Sonication was performed on ice with a Bronwill Biosonik unit, equipped with a microprobe, at a power setting of 80 for two 30-s periods separated by a 60-s period of cooling. Homogenates were kept on ice for at least 30 min and centrifuged at 31,000 × *g* for 45 min at 4°C. Re-extraction of the pellet with TCA only increased the recovery of [^{3}H]Ap_4A by about 5%. Consequently, re-extraction was not routinely done. In preliminary experiments acid-soluble extracts were prepared by homogenizing macroplasmodia in a Potter-Elvehjem homogenizer with a Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) pestle. Levels of Ap_4A and recoveries of [^{3}H]Ap_4A were similar to those for extracts prepared by sonication.

Sample fractionation. Samples to be assayed by HPLC were purified as described previously in detail (17). After the addition of acetic acid to raise the salt concentration to 1 M, the extract was neutralized with NH_4OH. When ATP was to be assayed, 0.1 ml was removed at this point. The pH was then adjusted to 9.8, and an equal volume of cold ethanol was added to precipitate plasmodial slime material. The ethanolic supernatant fraction was subjected to boronate chromatography to isolate a fraction containing predominantly Ap_4A and other dinucleoside polyphosphates. This chromatographic procedure completely removed salts and TCA. The dinucleotide fraction was treated with alkaline phosphatase to remove residual mononucleotides and then subjected to HPLC analysis.

Samples to be assayed by the luciferase method were purified by a modification of a described procedure (39, 50). The acid supernatant fractions were extracted with an equal volume of 0.5 M tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane to remove the acid (11). The upper phase was brought to 60% (vol/vol) ethanol and kept on ice for 30 min, and the precipitate was removed by centrifugation. The ethanol was removed, and the volume was reduced to less than 3 ml by rotary evaporation on a manifold at 35°C. Samples were made 10 mM in Tris, and the pH was adjusted to 8.5. They were then treated with 0.5 U of *E. coli* alkaline phosphatase for 8 h at 25°C. An Ap_4A fraction was prepared by DEAE-cellulose chromatography as described previously (39, 50), lyophilized, and dissolved in 1 ml of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.5). Samples were then treated with 0.5 U of alkaline phosphatase for 3 h at 25°C.

Assay of Ap_4A and Ap_4G. Ap_4A and Ap_4G were assayed in some samples by isocratic, anion-exchange HPLC as described previously (17), except that the A and B buffers were 0.135 M ammonium phosphate (pH 5.5) and 0.30 M ammonium phosphate (pH 5.5), respectively. These conditions were chosen to optimize the separation of Ap_4A and Ap_4G. The Ap_4A eluate from the HPLC column was collected and counted in a liquid scintillation counter (Beckman LS 7000 or LS230 instrument) to determine recovery of each sample. Recoveries were generally 45 to 60%. The recovery of Ap_4G was assumed to be equal to that of Ap_4A.

In some samples Ap_4A was assayed by luciferase detection of the ATP released by phosphodiesterase I. The procedure described here was adapted from published methods (34, 39). Luminescence was measured in a Beckman LS230 scintillation counter with the coincidence circuit switched off (48). A sample (0.5 ml) was mixed with 0.2 ml of luciferin cocktail [75 mM Tricine (15 mM), mM $Mg(C_2H_3O_2)_2$, 1.5 mM

EDTA, 1.5 mM dithiothreitol, 4.5 mg of bovine serum albumin per ml, 37.5 mM Na_2HPO_4, 9 mM NaN_3, 15 μM Na_4PP_i, 90 μM synthetic D-luciferin (pH 7.7)]. Pure firefly luciferase was made up at 1 mg/ml in 0.5 M Tricine–3 mM NaN_3–90 μM D-luciferin and diluted in luciferin cocktail at the time of the assay. Luciferase dilution was adjusted to give a suitable level of counts for the desired range of sensitivity. Generally, 10 to 20 μl of a 50- to 250-fold dilution was used. After the addition of luciferase a background reading was taken (0.5 min after a 2-min equilibration time). Phosphodiesterase I (2 μg in 20 μl) was then added, and the kinetics of ATP release were monitored by repetitive counting. The peak magnitude was a linear function of the amount of Ap_4A over a 1,000-fold range. The use of a standard curve was impractical, since at the dilution used in this study luciferase activity decreased significantly over a period of hours. Quantitation was done by assaying half of each sample by the addition of an Ap_4A internal standard. The response to the additional Ap_4A was used to determine the amount of endogenous Ap_4A. The use of internal standard also compensated for the possible presence of luciferase-inhibiting substances in samples. For recovery determination the two halves were pooled, the luciferase reaction was stopped with 50 μl, of 1.0 M acetic acid, and [^{3}H]Ap_4A was determined by liquid scintillation counting. Recoveries were generally 40 to 60%.

Determination of ATP. ATP was measured by a luciferase assay similar to that for Ap_4A. Sample (20 μl of a 50-fold dilution in 10 mM Tricine [pH 7.7]), 0.2 ml of luciferin cocktail (the same as that used for Ap_4A, except that $Na_2H_2PO_4$ was omitted), and 0.5 ml of 10 mM Tricine (pH 7.7) were mixed; and the reaction was started by the addition of 20 μl of a 1,000-fold dilution of luciferase in luciferin cocktail. The solution was counted for 12 s after a 75-s delay. Internal standard ATP (an amount comparable to the endogenous ATP) was then added, and the solution was recounted. Endogenous ATP was quantitated based on the increment in counts given by the internal standard. Each sample was assayed in duplicate.

Determination of [^{3}H]thymidine incorporation and protein. Acid-insoluble pellets were washed twice with 95% (vol/vol) ethanol, and nucleic acids were hydrolyzed by incubation at 80°C in 0.5 M $HClO_4$ for 20 min. Supernatant fractions were counted for ^{3}H, and the pellets were dissolved in 2 M NaOH and assayed for protein by the method of Lowry et al. (30), using bovine serum albumin as the standard. Samples which were assayed for Ap_4A by the luciferase method were not labeled with [^{3}H]thymidine because the purification was inadequate to separate thymidine metabolites from the final Ap_4A fraction.

Data analyses. Data values represent the mean and range of analysis from two individual plasmodia at each time point of collection. All experiments were done a minimum of two times, and representative data are presented. The data are expressed as picomoles of Ap_4A (or Ap_4G or ATP) per milligram of protein. To compare these values to values reported previously as concentrations, the wet weight, the dry weight, and the protein relationships were determined. Macroplasmodia from *P. polycephalum* M_3CVII were weighed in tared vials before and after they were dried at 110°C for 24 h. The protein content of the dried plasmodia was determined by the method of Lowry et al. (30) after dissolution in 2 N NaOH. The values were 0.10 ± 0.005 g (dry weight) per 1.0 g (wet weight) and 281 ± 13 mg of protein per g (dry weight) ($\bar{x}$ ± standard deviation; n = 7). There were no significant differences in these relative values among macroplasmodia collected at different phases of the cell cycle.

TABLE 1. Comparison of different fixation methods for *P. polycephalum* macroplasmodia

Expt	Method	Ap_4A (pmol/mg protein) at [a]:	
		G_2 phase	S phase
1	A	0.68 ± 0.04 (3)	0.32 ± 0.02 (3)
	B	0.55 ± 0.05 (2)	0.24 ± 0.02 (3)
	C	0.90 ± 0.10 (3)	0.73 ± 0.03 (3)
2	C	0.43 ± 0.02 (3)	0.48 ± 0.02 (3)
	D	0.50 ± 0.02 (3)	0.51 ± 0.03 (3)
3	D	1.11 ± 0.01 (2)	1.17 ± 0.01 (2)
	E	0.63 ± 0.05 (2)	0.69 ± 0.07 (2)
	F	0.98 ± 0.09 (2)	0.85 ± 0.12 (2)

[a] The effect of different macroplasmodium fixation procedures on the Ap_4A values measured was tested in three experiments. All samples were fractionated by APB-BioRex 70 chromatography and assayed by HPLC. The values given are the mean ± the standard error of the mean with the number of samples in parentheses. The collection methods are given in more detail in the text.

RESULTS

Tests of different methods of sample fixation. Adenine nucleotides are known to have in vivo turnover times of near 1 s (10). In previous studies Ap_4A was found to decrease at least as fast as ATP under conditions which decrease the cellular energy charge (38, 42). These facts suggest that collection procedures which differentially interfere with synthesis, degradation, or precursor (presumably ATP) supply result in rapid changes in Ap_4A levels. If synthesis is more sensitive than degradation, the procedure giving the highest values should be the best. This assumption is supported by the fact that in samples that were frozen and thawed before they were fixed in acid, Ap_4A decreased to undetectable levels (data not shown).

A number of sample collection procedures were tested to see whether the measured Ap_4A levels varied significantly (Table 1). Within each experiment, procedures in which the the plasmodium was rapidly dispersed in acid (methods C and F) gave relatively higher values, while procedures which involved freezing and thawing into acid (methods A and B) or a longer time until dispersal in acid (method E) gave lower values. When frozen samples were lyophilized before they were treated with acid (method D), reactivation of degradative enzymes was apparently avoided and higher values were obtained. Qualitatively similar results were obtained for Ap_4G. Methods C and D were chosen to be used in cell cycle experiments. Method D was used in experiments on oxidative stress.

Ap_4A and Ap_4G measurements during the cell cycle. Measurements of Ap_4A and Ap_4G by HPLC and Ap_4A by the luciferase assay in parallel sets of plasmodia are shown in Fig. 1 and 2. Collection methods D and C were used (Fig. 1 and 2, respectively). No cell cycle-specific changes in either of the compounds were detected. Similar results were obtained in two preliminary experiments by a collection method similar to method B, homogenization, and the HPLC assay (data not shown). In one experiment (Fig. 1) two luciferase-assayed samples, one in mitosis and one in the S phase, showed elevated Ap_4A levels. However, these points were not matched by their duplicates or by the HPLC-

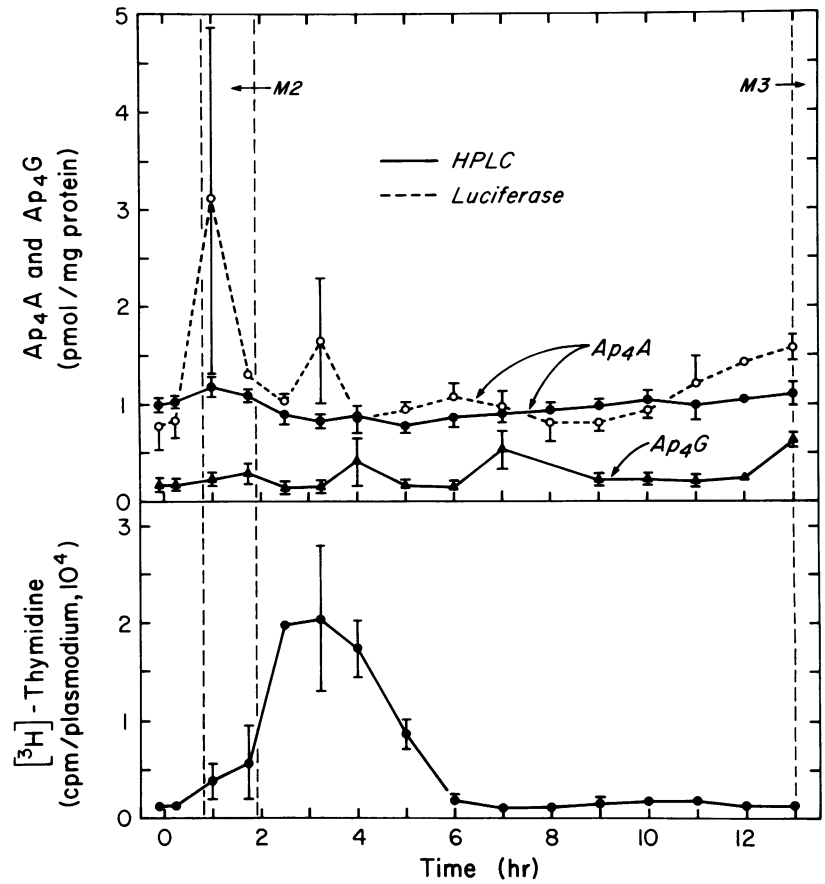

FIG. 1. Ap_4A and Ap_4G levels during the cell cycle of *P. polycephalum* with macroplasmodia collected by freezing in liquid N_2-cooled isopentane, lyophilizing, and sonicating in cold 5% TCA (method D; see text). Four plasmodia were collected at each time point, and two were assayed by each method (HPLC and luciferase). Only plasmodia assayed by the HPLC method were labeled with [^{3}H]thymidine. Values plotted represent duplicate plasmodia and the mean. M2 and M3 represent the second and third mitosis postfusion, respectively.

assayed samples. In another experiment (Fig. 2) the luciferase-assayed samples gave higher values for Ap_4A than the HPLC-assayed samples, but again neither the higher values nor the difference seemed to be cell cycle specific. Occasional elevated values were also seen for Ap_4G (Fig. 1), but these also were not reproducible (Fig. 2). In one experiment, macroplasmodia were collected by method C at 15-min intervals from just prior to the second mitosis through the S phase, and Ap_4A was measured by the HPLC procedure. The levels of Ap_4A were about 1 pmol/mg of protein throughout mitosis and the S phase.

The luciferase assay is not expected to be absolutely specific for Ap_4A, since ATP should be released from any Ap_4N compound by phosphodiesterase I. The luminescence reaction was found to be very specific for ATP, giving <0.5% of the ATP response with CTP, dATP, and Ap_4A and about 4% with GTP. These values are similar to those found by Moyer and Henderson (33). Ap_4N compounds were expected to give a 50% response with phosphodiesterase I addition. However, Ap_4G gave only a 28% response, suggesting that the phosphodiesterase may prefer the GTP-releasing cleavage. The luciferase assay therefore measures a combination of several compounds, but since Ap_4A gives the greatest assay response and is generally present in the largest amount as measured by the HPLC assay, the luciferase assay is probably an accurate measure of its concentration. The ATP-releasing material in extracts of *P. polycephalum* was completely destroyed by Ap_4A pyrophosphohydrolase from the same organism, an enzyme which is very specific for dinucleoside polyphosphate compounds (18).

The cell cycle times in Fig. 1 and 2 are long compared with the fastest times reported in *P. polycephalum* (7 to 8 h) (32). If Ap_4A is a positive regulator related to growth, it is possible that higher levels or a definite cell cycle pattern would be seen with shorter cycle times. However, when a shorter cycle time (9.5 h) was obtained by first passing the organism (strain M_3CVII) through the spherule phase, no difference was seen between G_2 and S phase (Table 1, Experiment 3). Similar results were obtained with strain M_3CVIII, which had cycle times of 8 to 9 h (data not shown). Based on analysis of about 200 macroplasmodia in seven cell cycle experiments, there was no reproducible, cell cycle-specific change of either Ap_4A or Ap_4G in *P. polycephalum*.

Measurement of Ap_4A, Ap_4G, and ATP during DNP treatment. DNP (0.1 mM) treatment for 1 h increased the levels of Ap_4A and Ap_4G by three- to sevenfold (Fig. 3A and B). Although starvation on salts medium enhanced the responses of Ap_4A and Ap_4G to DNP, starvation alone did not result in significant increases in either dinucleotide, either at early times after the shiftdown (data not shown) or during the time period of the experiment (Fig. 3). There was no evidence by microscopic examination for formation of spherules (sclerotia) under these starvation conditions at

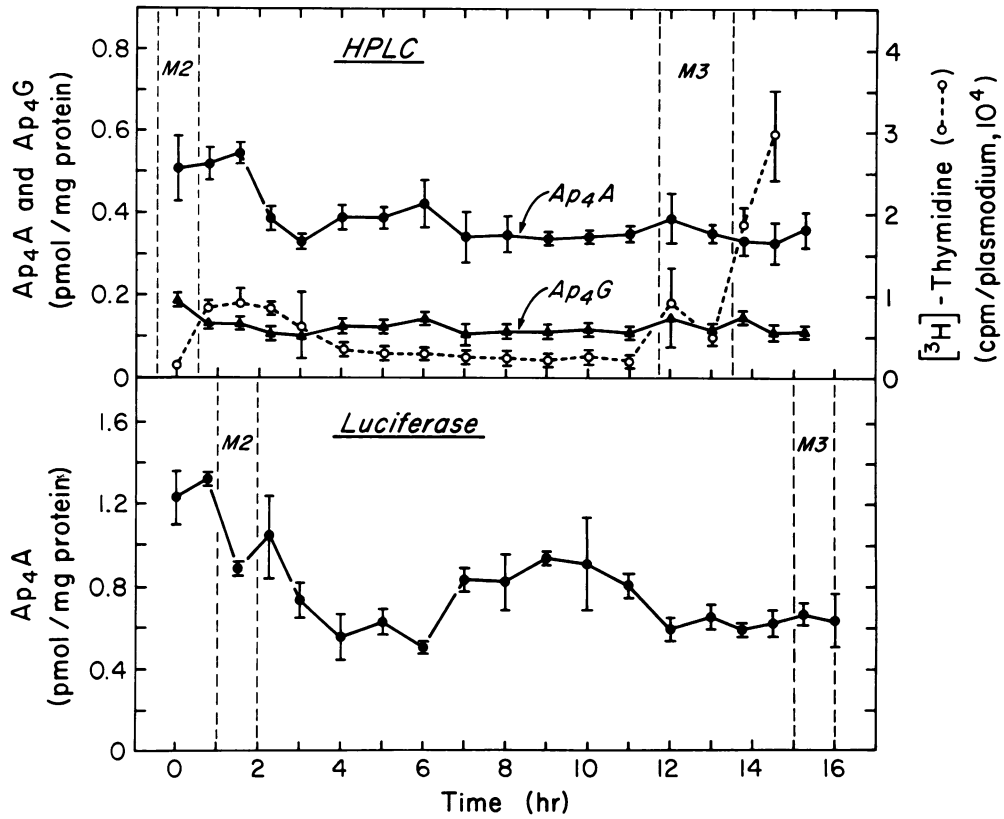

FIG. 2. Ap_4A and Ap_4G levels during the cell cycle of *P. polycephalum* with macroplasmodia collected by scraping into cold 5% TCA and sonicating (method C; see text). Parallel sets of plasmodia were prepared 30 min apart. The first set was collected for assay by HPLC, and the second set was collected for assay by the luciferase assay. Results of the two assays are plotted separately because of the different observed times for mitosis. Only plasmodia assayed by HPLC were labeled with [^{3}H]thymidine. Values plotted represent duplicate sets of plasmodia and the mean.

these or later times. Although there were measurable changes in the protein content of the plasmodia during the course of the experiment (20% increase in fed controls; 10 to 25% decrease in starved or DNP-treated plasmodia or both), they were not sufficient to account for the measured changes in dinucleotide concentrations.

The decreases in Ap_4A and Ap_4G in DNP-treated macroplasmodia after the peak at 1 h corresponded to the decrease in ATP to very low levels (Fig. 3C), which is consistent with the probable role of ATP in the synthesis of the dinucleotides. The peak of ATP at 2 h in the fed controls probably corresponds to the early mitotic peak of ATP reported by others (5, 15, 46), since DNA replication, as measured by [^{3}H]thymidine incorporation, began in the fed controls near that time (data not shown). (There is no G_1 phase in *P. polycephalum*.) The maximum level of about 40 nmol of ATP per mg of protein reported here may be compared with maximum levels of 8 to 18 nmol of ATP per mg of protein reported previously (5, 15, 46).

Macroplasmodia in growth media exhibited the normal S-phase replication of DNA. The S phase was not detected in the starved macroplasmodia (data not shown). This is consistent with the starvation-induced delay of mitosis reported by others (23, 49). DNP treatment eliminated within 1 h the G_2-phase thymidine incorporation (rDNA and mitochondrial DNA synthesis [25, 56]) in plasmodia on either medium, and the S phase was likewise not detected (data not shown).

The method of fixation of macroplasmodia was critical to detect maximum levels of Ap_4A, Ap_4G, and ATP during oxidative stress. The concentrations of the nucleotides in macroplasmodia in growth media with DNP for 1 h were 2.7 ± 0.1 pmol of Ap_4A, 0.87 ± 0.13 pmol of Ap_4G, and 25.4 ± 1.5 nmol of ATP per mg of protein after fixation by method D. The concentrations in macroplasmodia fixed by method C were decreased by 44% to 1.5 ± 0.13 pmol of Ap_4A, by 33% to 0.58 ± 0.03 pmol of Ap_4G, and by 88% to 2.9 ± 2.3 nmol of ATP per mg of protein. Similar percent differences from the different methods of fixation were detected for macroplasmodia in starvation media with DNP for 1 h.

DISCUSSION

Based on our measurements of Ap_4A and Ap_4G in *P. polycephalum*, there are no reproducible, cell cycle-specific changes of either nucleotide in this organism. Clearly, changes in levels of Ap_4A and Ap_4G are not required for traversing the cell cycle. The results presented here for the level of Ap_4A during the cell cycle of *P. polycephalum* are in contrast with those recently reported by Weinmann-Dorsch et al. (50, 51). They reported a level at mitosis of about 0.1 μM Ap_4A which increased by 8- to 30-fold in early S phase, decreased to an intermediate value in late S phase, and slowly decreased during the G_2 phase to a level seen at mitosis (51). (They expressed Ap_4A levels as concentrations assuming that 1 g [wet weight] is equal to 1 ml [50]). Our basal value of 0.5 to 1.0 pmol of Ap_4A per mg of protein is equal to about 0.015 to 0.03 μM (see above). Since our values were about one-third to one-sixth of the values

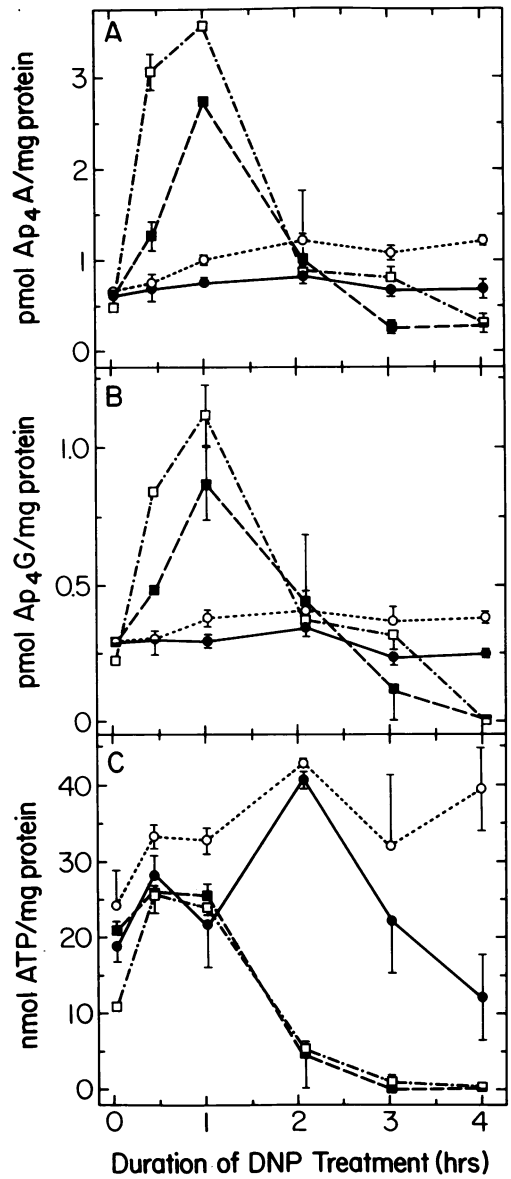

FIG. 3. Ap_4A, Ap_4G, and ATP levels in *P. polycephalum* as a function of treatment with DNP. Macroplasmodia were maintained on growth medium or shifted to salts medium at 40 min after the second mitosis. DNP was added to a final concentration of 0.1 mM to the medium of macroplasmodia at 8.3 h after the second mitosis. [^{3}H]thymidine (10 μCi) was added to the medium of each macroplasmodium 25 min before collection. Macroplasmodia were collected at the indicated times after treatment by freezing in liquid N_2-cooled isopentane, lyophilizing, and sonicating in cold 5% TCA (method D; see text). The cell cycle duration was about 10 h in control plasmodia. Symbols: ●, growth medium, no DNP; ○, salts medium, no DNP; ■, growth medium plus DNP; □, salts medium plus DNP. Values plotted represent duplicate sets of plasmodia and the mean. (A) Values for Ap_4A; (B) values for Ap_4G; (C) values for ATP as a function of time of treatment with DNP.

reported by Weinmann-Dorsch et al. (51), one possible explanation for the differences would be an inadequate fixation procedure. However, given the number of methods that we have tried with similar results, we believe that this is unlikely. This view is supported by the fact that Weinmann-Dorsch et al. used a collection procedure essentially identical to method C except without sonication (51). In addition, our data on Ap_4A, Ap_4G, and ATP concentrations with different methods of fixation of macroplasmodia treated with DNP indicate that ATP is more labile than Ap_4A and Ap_4G in *P. polycephalum*. Yet our basal ATP concentrations were equal to or greater than reported ATP concentrations in *P. polycephalum* (5, 15, 46).

Two types of assay for Ap_4A have been employed in these studies. Weinmann-Dorsch et al. (51) used a method based on isolation of Ap_4A by anion-exchange chromatography and detection by a coupled phosphodiesterase-luciferase assay. In initial experiments we used the boronate chromatography-HPLC method. When no changes were detected by that method, we then did parallel measurements with the two assays to see if a difference in the assays might account for the different results. This possibility was considered because the specificities of the two assays are not identical. The luciferase assay detects any Ap_4N compound which can be hydrolyzed by phosphodiesterase I to yield ATP. The DEAE-cellulose column purification is not selective, and it is likely that any such Ap_4N compound would be present in the Ap_4A fraction. The HPLC assay detects Ap_4N compounds as separate peaks. (One or two such peaks beside Ap_4A and Ap_4G are regularly seen but show no change during the cell cycle). However, the Ap_4N structure must have two *cis*-diol groups or it will be lost during the boronate chromatography used for purification of samples for the HPLC assay. Thus, structures of the type Ap_4dN would be detected by the luciferase assay, but not by the HPLC assay. (Structures such as dAp_4dN were not detected by either assay, since the luciferase requires ribo-ATP). Structures of all these types have been reported as in vitro products of the Ap_4A-synthesizing tRNA synthetases (40). It was therefore possible that discrepancies between the two assays could be due to the contributions of multiple dinucleoside polyphosphates. However, since we did not detect a reproducible S-phase peak by either method, this cannot be the explanation for the different results.

Despite the lack of a cell cycle-specific pattern of Ap_4A levels, a role in cell cycle regulation cannot be completely excluded. Possible mechanisms for such a role include compartmentalized nucleotide pools and varying fluxes of Ap_4A. If the Ap_4A concentration was only elevated in a small locale (e.g., at the nucleus or at replication origins), a change in the average concentration might not be observable. Weinmann-Dorsch et al. (51) have reported that Ap_4A is elevated in isolated S-phase nuclei. Another possibility is that Ap_4A might serve a substrate function rather than that of an allosteric effector, in which case the flux could change with no observed change in concentration. These possibilities are of interest in view of the postulation that Ap_4A could serve as a primer for DNA replication (53) and the observed association of certain tRNA synthetases (9, 44) and other nucleotide-synthesizing enzymes with DNA polymerase α (45). If either compartmentalization or varying fluxes of Ap_4A were involved in its function, it is possible that different patterns of change in the average concentration could be seen under different conditions, while Ap_4A was in fact serving a regulatory role in the cell cycle.

While the mechanisms mentioned above are possible, the occurrence of drastically different levels and patterns of Ap_4A, as reflected by our results in comparison with those of Weinmann-Dorsch et al. (51), in apparently normal *P. polycephalum* cell cycles casts doubt on a primary role of Ap_4A as a trigger of DNA replication or other cell cycle events. Some previously reported data support this conclu-

sion. For example, Ap_4A levels increased over 40-fold after *T. pyriformis* was shifted to a starvation salts medium (37). Ap_4A levels in simian virus 40-transformed 3T3 cells were the same as those in normal 3T3 cells (2). Ap_4A did not stimulate DNA synthesis in permeabilized, normal human fibroblasts (14) under conditions used to demonstrate Ap_4A stimulation of DNA synthesis in baby hamster kidney cells (21). These reports should be balanced against reports cited earlier in this report that suggest that the role of Ap_4A in DNA replication is via a mechanism of fluctuating cellular levels. However, Ap_4A could be involved in DNA replication in some manner. The occurrence of an Ap_4A-binding subunit of DNA polymerase α has been reported by three groups that used two different sources of polymerase (3, 22, 41). The relevance of this Ap_4A-binding subunit in DNA replication remains to be established.

We detected transient increases of three- to sevenfold in Ap_4A and Ap_4G in macroplasmodia treated with DNP. Plesner and Kristiansen (37) previously reported a large increase of Ap_4A in *T. pyriformis* after a downshift to starvation medium. The same increase occurred more rapidly when 0.5 mM DNP was present in the starvation medium. We found that in *P. polycephalum* starvation alone did not cause any change in the nucleotides.

The mechanism of the DNP effect on Ap_4A and Ap_4G is uncertain. DNP at the concentration employed (0.1 mM) is an effective uncoupler of oxidative phosphorylation in mitochondria from *P. polycephalum* (26) and *Physarum flavicomum* (1). The uncoupling of respiration in mitochondrial preparations generally decreases the production of H_2O_2 and O_2^- (16). However, production of H_2O_2 by antimycin-treated mitochondria is stimulated by uncouplers (8). Mitochondria from DNP-treated rats showed a 60-fold increase in production of H_2O_2 over that from controls, suggesting that a similar effect may occur in vivo (13). If such an in situ effect occurred in our experiments, the results would be consistent with the proposal that oxidative stress triggers increased levels of Ap_4A and Ap_4G (7). It is possible however, that nonmitochondrial effects of DNP could be involved in the changes in Ap_4A and Ap_4G. That such effects may be present is suggested by the fact that [^{3}H]thymidine incorporation was blocked considerably before any effect on the ATP concentration (data not shown).

Determination of the mechanism of the DNP-triggered increases in Ap_4A and Ap_4G and their relation to oxidative stress will require further study, but the changes measured indicate that if changes in Ap_4A and Ap_4G had occurred in the cell cycle experiments, the method employed would have detected them. The fact that Ap_4A increases under conditions which are so strongly inhibitory to growth also argues against any role for it as a positive pleiotypic activator.

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