THE STRUCTURE OF CHROMATIN SYNTHESIZED IN THE PRESENCE OF CYCLOHEXIMIDE IN PHYSARUM POLYCEPHALUM

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The effect of cycloheximide on protein and DNA synthesis and on the structure of chromatin was studied. Changes in the rate and extent of DNA synthesis in response to cycloheximide were highly variable in contrast to the extremely rapid and reproducible inhibition of protein synthesis. No differences in the rate of the release of acid-soluble products by nucleases and in the nature of the nucleoprotein particles were found in chromatin from plasmodia treated and non-treated with cycloheximide. It is concluded that, in Physarum polycephalum, unlike in higher animals, chromatin from the antibiotic-treated plasmodia is structurally indiscernible by the methods applied from normal chromatin.

Studies on chromatin biogenesis have demonstrated that this process is closely linked—both temporally and functionally—with DNA and protein synthesis. Generation of the nucleohistone complex so characteristic for eukaryotic organisms takes place almost immediately with the short stretches of DNA already formed. Nascent DNA, however, although found in nucleosomes, is more susceptible to nucleases than DNA in mature chromatin. This higher susceptibility of newly synthesized DNA lasts only for a few minutes, then changes, most likely conformational, make "new" nucleosomes indiscernible from bulk chromatin (Seale, 1978). Therefore, despite basic similarity of DNA polymerization in pro- and eukaryota, in the latter additional processes probably coordinate replication and formation of the basic structural element of chromatin.

It is well established (Sheinin et al., 1978) that DNA replication in eukaryotes depends on concomitant protein synthesis. Studies with inhibitors of protein synthesis (mainly cycloheximide) and isolated nuclei (where no protein synthesis occurs) have shown, however, that this dependence is not
absolute. In the absence of protein synthesis, replication continues although at a reduced rate and for a limited period of time. The question therefore arises whether the DNA formed during this residual synthesis is “packed” into normal chromatin, or, if chromatin with an altered structure is formed. Seale & Simpson (1975) and Seale (1976) demonstrated that, in HeLa cells, the chromatin produced in the absence of protein synthesis is significantly different from normal chromatin. Similar reports have been made for chick erythroblasts (Weintraub, 1976) and a Chinese hamster lung cell temperature-sensitive mutant (CHL ts14), (Roufa, 1978). However, our previous data (Jerzmanowski & Toczek, 1973) suggest that chromatin synthesized under conditions of limited protein synthesis in pea root tips shows the same sensitivity to DNAase I as normal chromatin.

In Physarum polycephalum inhibition of DNA replication by cycloheximide has been convincingly demonstrated by Funderud & Haugli (1977). No attempts have been made, however, to analyse the structure of the newly synthesized chromatin. In this report we show that in P. polycephalum, in contrast to most of the other eukaryotic cell lines studied, the structure of the chromatin synthesized in the presence of cycloheximide is indiscernible from that of normal chromatin.

MATERIALS AND METHODS

Cultures. P. polycephalum, strain M₃IV, was maintained in submerged, shaken culture as described by Daniel & Baldwin (1964).

Incubation procedure. Plasmodia were harvested by centrifugation at 50 g for 1 min and rinsed twice with 10 vol. of salt solution (0.3 mM-CaCl₂, 0.25 mM-MgSO₄, 0.04 mM-MnCl₂, 0.01 mM-ZnSO₄, pH 4.6). They were then suspended in the same solution at a density of 10-20 A₄₁₅ at 26°C. Cycloheximide and isotopes were added at the time and concentration indicated in the text (see Results) in a volume which made up 1/3 of the final volume of the incubation mixture. An equal volume of salt solution but without cycloheximide was added to control mixtures. Plasmodia were quickly and thoroughly mixed with the use of a very loose-fitting glass pestle, immediately after addition of any solution.

Incubation procedure. Plasmodia were harvested by centrifugation at 50 g suspended in 50 vol. of ice-cold buffered sucrose (0.01 mM-Tris/HCl, pH 7.8, containing 0.25 mM-sucrose, 0.01 mM-MgCl₂, and 0.1% Triton X-100) and homogenized in a blender for 2×30 s at 90 V and 2×15 s at 210 V. All steps were done on ice. The homogenate was filtered through four layers of cheese-cloth and sedimented by centrifugation for 20 min at 1400 g. The pellet was resuspended in the same buffer, layered over 2 mM-sucrose, 0.01 mM-Tris/HCl, pH 7.8, 0.01 mM-MgCl₂, 0.1% Triton X-100, and centrifuged for 20 min at 1400 g. The nuclear pellet was then washed twice by suspension and centrifugation in the appropriate digestion buffer (see below).
Digestion of nuclei. Nuclei were suspended at a concentration of 10 - 15 A_{260}^{\text{cm}} in a digestion buffer containing 0.25 m-sucrose, 1 mm-Tris /HCl, pH 7.8, and either 0.1 or 1.0 mm-MgCl$_2$ at 37°C. Micrococcocal nuclease or DNAase I was added to the final concentration indicated in the text. The reaction was stopped by either procedure $A$ or $B$ described below:

$A/ 0.1$ m-NaEDTA was added to a final concentration of 0 mm and the mixture chilled on ice. The digest was then centrifuged at 0°C for 20 min at 14,000 g. The supernatant was used for further studies.

$B/ 500$ $\mu$l aliquots were added to 250 $\mu$l of 1.5 m-HClO$_4$ and chilled on ice. After centrifugation for 10 min at 1400 g, the supernatant was used directly for radioactivity assay. The precipitate was hydrolysed in 750 $\mu$l 0.5 m-HClO$_4$ at 80°C for 30 min and used for radioactivity counting in Unisolve 1. This method was used to determine the percentage of acid-soluble products.

Chromatography. After digestion of nuclei with either micrococcocal nuclease or DNAase I the reaction was stopped by procedure $A$. The supernatant was transferred to a dialysis bag and concentrated against sucrose approximately 10-fold. This procedure removes most small DNA fragments not complexed with protein (acid-soluble products). A 200 $\mu$l portion of this concentrated digest was applied directly to a column, or NaCl was added to a final concn. of 2.0 m, and the solution was kept overnight at 4°C before chromatography. Biogel A 5M 100 - 200 mesh (0.4 x 50 cm) columns equilibrated with 0.01 m-Tris/HCl, pH 7.8, containing 5 mm-NaEDTA and either 0.5 or 2.0 m-NaCl as indicated, were used. The column was eluted with the same buffer at a rate of approximately 0.15 ml/min. Fractions (0.4 - 0.5 ml) were collected and used for radioactivity assay. In the case of the eluent containing 2.0 m-NaCl, 500 $\mu$l of water were added before addition of Unisolve 1.

Determination of DNA and protein synthesis. Portions of the incubation mixture (about 0.2 ml) were added to 3 ml of 5% (w/v) trichloroacetic acid (TCA) in 50% aqueous acetone. The precipitate was washed twice with the TCA-acetone mixture and twice with 3 ml of 0.25 m-HClO$_4$, dissolved in 0.1 m-NaOH and used for protein and radioactivity assay. Samples were counted in Bray’s solution (1960). Isotope incorporation was expressed as: c.p.m. $^{14}$C or $^3$H per mg protein. The protein content was determined by the method of Lowry et al. (1951).

DNA content in isolated nuclei was determined by the method described by Burton (1956), and incorporation of $^3$H]thymidine assessed by counting in Unisolve 1. Samples were prepared as follows: after alkaline hydrolysis in 0.3 m-KOH at 37°C and precipitation by 1.2 m-HClO$_4$, the pellet was hydrolysed in 0.5 m-HClO$_4$ for 30 min at 80°C. After centrifugation, the supernatant was used for radioactivity and DNA assay.
Reagents. Cycloheximide and Triton X-100 were obtained from Serva (Heidelberg, F.R.G.); [3H]thymidine (25 Ci/mmol) from UVVVR (Prague, Czechoslovakia); [14C]glycine (13 Ci/mmol) from Opdi (Swierc, Poland); Unisolve 1 from Koch-Light (Colnbrook, England); DNAase I from Calbiochem (Los Angeles, Calif., U.S.A.); micrococcal nuclease from Worthington (Freehold, N.J., U.S.A.); Biogel A 5M and A 1.5M from BioRad Laboratories (München, F.R.G.). Other chemicals used were of analytical grade.

RESULTS

Protein and DNA biosynthesis. Figure 1 shows the typical pattern of incorporation of radioactive precursors into protein and DNA by plasmodia prepared as described in Methods. Incorporation was essentially linear for 60 min for both protein and DNA, and then gradually slowed down. The incorporation pattern was reproducible when expressed as a percentage of the total incorporation, up to 120 min, whereas absolute values (c.p.m. per mg protein) showed rather large variations. Differences in the biosynthetic capabilities of plasmodia in various experiments were insignificant in so far as sensitivity to cycloheximide was concerned (see below).

Fig. 1. Incorporation of [14C]glycine (A) and [3H]thymidine (B) by plasmodia of P. polycephalum. Preparation of plasmodia, determination of protein and DNA synthesis were as described in Methods. Averages of 6 (A) or 5 (B) experiments are shown. Concentration of [14C]glycine ranged from 7 to 9 µCi/ml, and that of [3H]thymidine, from 7 to 11 µCi/ml.

The effect of cycloheximide on protein and DNA biosynthesis. Figure 2 shows the average inhibition of biosynthesis attained with different concentrations of cycloheximide. Maximum inhibition of both protein and DNA biosynthesis was reached at 5 µg cycloheximide per 1 ml and remained at the same level at higher cycloheximide concentrations; inhibition of protein synthesis attained under these conditions ranged usually from 75% to 95%, in agreement with the results reported by others (Nations et al., 1974;
Cummins & Rusch, 1966). This is interpreted as possibly due to continued mitochondrial protein synthesis and incorporation of [14C]glycine into non-protein acid-insoluble compounds. In all of the following experiments, the concentration of 10 μg/ml cycloheximide was used.

Fig. 2. Effect of cycloheximide concentration on protein (♀) and DNA (△) biosynthesis. Bars mark the average standard deviation. Plasmodia were preincubated with cycloheximide for 10 min at 26°C, then [14C]glycine or [3H]thymidine was added (10 μCi/ml) and incubation continued for 60 min. Incorporation was determined as described in Methods.

Figures 3 and 4 show the temporal effects of cycloheximide on protein and DNA biosynthesis, respectively. The results presented for protein synthesis are typical for all experiments performed. Inhibition was observable immediately, reaching its maximum level within 5 min. Quite different effects were seen on inhibition of DNA replication. Figure 4 illustrates the differences in the inhibitory effect of cycloheximide on DNA synthesis; Fig. 4B represents the most typical results. In each case, inhibition of protein synthesis was practically complete (cf Fig. 3). The time lag between administration and the first visible effects of cycloheximide on replication ranged from 5 min (Fig. 4A) to 70 min (Fig. 4C). This suggests that a pool of replication proteins, variable in size, may be involved. In order to see whether the size of this possible pool depends on the handling of the plasmodia, we tested the effect of washing on the biosynthetic capability of plasmodia. It was found that only the first washing with salt solution reduced the overall biosynthetic activity; further washings, up to the 6th, were ineffective. Sensitivity to cycloheximide, however, remained on the same level in all cases (results not shown).

Susceptibility to nuclease digestion. The foregoing results showing that, in P. polycephalum, DNA synthesis may continue during complete inhibition of protein synthesis brought out the question whether this DNA was packed
Fig. 3. Effect of cycloheximide on protein biosynthesis. Plasmodia were preincubated with \(\text{[}^{14}\text{C}]\)glycine (10 \(\mu\text{Ci/ml}\)) for 20 min; one part of the mixture was transferred to the salt solution (○, control), and another to the solution containing cycloheximide at a final concentration of 10 \(\mu\text{g/ml}\) (□), and incubation was continued. Samples were withdrawn in triplicate, starting after addition of cycloheximide. For details see text.

Fig. 4. Effect of cycloheximide on DNA biosynthesis. Plasmodia were preincubated for 30 min (A) or 20 min (B and C) with \(\text{[}^{3}\text{H}]\)thymidine (10 \(\mu\text{Ci/ml}\)). Inhibition of protein biosynthesis after 10 min was 100, 97 and 96\%, respectively, for A, B and C. Plasmodia: control (○) and cycloheximide-treated (□).

into a chromatin with a normal or altered nucleosomal structure. To distinguish the chromatin synthesized in the absence of protein synthesis from normal chromatin, we first preincubated plasmodia with cycloheximide for 30 min, to be sure that the assembly of the chromatin structure
typical for conditions of protein inhibition was well under way, then added radioactive thymidine for 1 h to both control and cycloheximide-treated plasmodia. Susceptibility to micrococcal nuclease and DNAase I, and the chromatographic behaviour of the generated nucleoprotein particles from chromatin of both control and cycloheximide-treated plasmodia were compared. The amount of DNA rendered acid-soluble by the two enzymes is indicative, among others, of the density of nucleosome spacing. According to Seale (1976) and Bellard et al. (1978), altered nucleosome conformation can result in more rapid accumulation of monomers released upon digestion with nuclease, which can be visualized by column chromatography. Figure 5 and Table 1 show that both the rate of release and total amount of acid-

![Fig. 5. Digestion of nuclei with micrococcal nuclease or DNAase I. Plasmodia were preincubated with cycloheximide (10 μg/ml) for 30 min (□); control plasmodia (○), with the same volume of the salt solution. [3H]Thymidine (10 μCi/ml) was then added to both, and incubation continued for 60 min. Nuclei were then isolated and digested with either micrococcal nuclease (50 u/ml), (A, B), or DNAase I (11 u/ml), (C, D). Acid-soluble products were determined as described in procedure B (see Methods). Concentration of Mg²⁺ in the digestion buffer (see Methods) was 0.1 mm in A and C, and 1.0 mm in B and D. The DNA concentration was 90 μg/ml, except in B (100 μg/ml).

-soluble products from these two types of chromatins were practically the same under each set of conditions used. Both enzymes showed slightly higher activities in 0.1 mm-Mg²⁺ although this did not affect the digestion pattern. All of the preparations listed in Table 1 were run on Biogel A 5M and on Biogel A 1.5M columns and, in the total of 34 separations, in no case did any significant differences between chromatin of control and the cycloheximide-treated plasmodia appear. Figure 6 shows the results
Table 1

Digestion of nuclei from control and cycloheximide-treated plasmodia by nuclease

Calculated as percent of total radioactivity incorporated into DNA. Plasmodia and nuclei were prepared as described in the text. Nuclei were digested in 0.1 mM-Mg$^{2+}$ buffer at 37°C under the conditions given in the Table. The reaction was stopped by adding NaEDTA and chilling on ice. Aliquots of 500 μl were taken to determine the percentage of acid-soluble products (procedure B, see Methods). The remaining digest was centrifuged and prepared for chromatography as described in Methods.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Enzyme and time of digestion</th>
<th>DNA in nuclei (μg per unit enzyme)</th>
<th>Inhibition of DNA synthesis (%)</th>
<th>Acid-soluble DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>micrococcal nuclease, 10 min</td>
<td>4.0</td>
<td>52</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>Cycloheximide-treated</td>
<td>nuclease, 10 min</td>
<td>3.4</td>
<td>52</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>micrococcal nuclease, 15 min</td>
<td>2.0</td>
<td>48</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>Cycloheximide-treated</td>
<td>micrococcal nuclease, 30 min</td>
<td>4.9</td>
<td>57</td>
<td>10.8</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>micrococcal nuclease, 60 min</td>
<td>4.9</td>
<td>57</td>
<td>11.4</td>
</tr>
<tr>
<td>6</td>
<td>Cycloheximide-treated</td>
<td>DNAase I, 5 min</td>
<td>19.0</td>
<td>57</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>DNAase I, 20 min</td>
<td>18.2</td>
<td>42</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Cycloheximide-treated</td>
<td>DNAase I, 20 min</td>
<td>19.0</td>
<td>42</td>
<td>19.6</td>
</tr>
</tbody>
</table>

of chromatography of these two chromatin digested by micrococcal nuclease and DNAase I on Bioigel A 5M in 0.5 mM-NaCl. Figure 7 shows two typical runs on the same column in 2.0 mM-NaCl. Under these conditions—of high ionic strength where no aggregation is possible, the chromatographic patterns were in all cases also practically identical. Although these conditions of chromatography do not allow separation of monomers from dimers etc., effective segregation according to molecular mass was achieved (Fig. 7). Meaningful differences in rates of monomer accumulation should, therefore, be distinguishable under these conditions. Thus, it can be concluded that the structure of the two chromatin is indiscernible by digestion with micrococcal nuclease and DNAase I.

DISCUSSION

The results presented in this paper show that the dependence of DNA replication on concomitant protein synthesis in P. polycephalum is less marked than in animal cells where cycloheximide was reported to immediately reduce by 80% the rate of DNA synthesis (Seale & Simpson, 1975). The different mode of reaction of P. polycephalum suggested at first that
the coupling of DNA replication with the assembly of the proper histone structure is not as stringent as in higher organisms. A consequence of this would be the production of a larger amount of histone-deficient chromatin than in HeLa cells. In this case, digestion with micrococcal nuclease and DNAse I—enzymes which are able to discriminate histone-free regions, should bring out such possible differences in Physarum. We have used various methods of analysis of digestion products of the two chromatin, and have not been able to find any differences. This means that nucleosome
Fig. 7. Biogel A 5 M column chromatography of digests of nuclei from control (○) and cycloheximide-treated (□) plasmodia with micrococcal nuclease (A) or DNAAse I (B). The conditions were as described for Fig. 6 except that the buffer contained 2.0 m-NaCl. The content of acid-soluble products in the control and cycloheximide-treated plasmodia was as follows: A. 3.8 and 3.5%; B. 19.6 and 16.0%, respectively.

Spacing and conformation are very similar, if not identical, in the chromatin from control and cycloheximide-treated plasmodia. Our results lead us to the conclusion that DNA synthesis in P. polycephalum is very closely controlled by the structure of the chromatin into which nascent DNA is packed. This control seems to be very stringent, much more so that in animal cells, where certain amounts of structurally different chromatin do arise before replication is halted by cycloheximide. For this reason, we think that in P. polycephalum a complete pool of replication proteins, including histones, must exist. The size of this pool is variable, as judged by the differences in the time of response of DNA synthesis to cycloheximide. However, even when replication was finally affected, chromatin of undisturbed structure was produced; replication was halted before structural changes, detectable by nuclease, took place. This very close coupling of DNA replication with generation of only normal chromatin structure in P. polycephalum signalizes the existence of mechanisms preventing formation of structurally altered chromatin.

REFERENCES


**STRUKTURA CHROMATYNY PHYSARUM POLYCEPHALUM POWSTAŁEJ W OBECNOŚCI CYKLOHEKSIMIDU**

**Streszczenie**

Zbadano wpływ cykloheksamidu na proces syntety białka i DNA oraz na budowę chromatyny powstałej w obecności tego inhibitora. Wykazano, że cykloheksamid hamuje w sposób powtarzalny syntezę białka natychmiast po jego dodaniu do plazmódii, natomiast w tych samych warunkach hamuje syntezę DNA w różnym stopniu i po różnych czasie. Wykazano analizę porównawczą podatności na nukleozyd chromatyny kontrolnej i chromatyny plazmódów poddanych działaniu cykloheksamidu. Nie znaleziono żadnych różnic zarówno w ilości i szybkości uwłaniania fragmentów DNA rozpuszczalnych w kwasie, jak i w budowie częścią nukleoproteinoidowych uwłaniających z obu typów chromatyny. Uzyskane dane dowodzą, że zahamowanie syntety białka i DNA przez cykloheksamid nie prowadzi do powstania u Physarum polycephalum chromatyny zmienionej strukturalnie, jak to obserwowano w przypadku komórek zwierząt.

Received 3 March, 1980.