Study of DNA Synthesis in Chromatin Isolated from HeLa Cells†

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ABSTRACT: When incubated in vitro, HeLa cell chromatin can synthesize DNA at a rate comparable to that observed with isolated nuclei. The in vitro DNA synthetic activity of chromatin reflects DNA synthesis in intact cells since chromatin from cells in S phase are several times more active than preparations derived from mitotic cells. The requirements for the synthesis of DNA by chromatin preparations are also similar to those of isolated nuclei and the size of the DNA pieces made in both systems is roughly comparable. The chromatin system offers several advantages not available with isolated nuclei. Chromatin will synthesize DNA for a much longer time than isolated nuclei so that larger amounts of DNA can be synthesized in vitro. In addition, although chromatin has its own endogenous ability to synthesize DNA, it is markedly stimulated by the presence of exogenously added HeLa cell DNA polymerase α, β, and γ, and, thus, may provide a new template system for the study of DNA synthesis.

DNA synthesis in eukaryotic cells is a complex process whose details are unknown. One of the approaches in the study of DNA replication has involved the use of isolated nuclei which perform a limited DNA synthesis in vitro and which are stimulated by addition of cytoplasmic factors (Friedman and Mueller, 1968). Isolated nuclei have also been used to study herpes, adeno, polyoma, and vaccinia viral DNA synthesis (Winnacker et al., 1972; Kolber, 1975; Van der Vliet and Sussenbach, 1972; LaColla and Weissbach, 1975) in vitro, but, as of yet, the limitations of DNA synthesis in the in vitro nuclear systems have curtailed their usefulness. Permeabilized (Seki et al., 1975, Berger and Johnson, 1976) or lysed cells (Tseng and Goulian, 1975), and cells subjected to microinjection procedures (Waqar and Huberman, 1975), have also been used for such studies. All of these systems have the possible disadvantages that membranes may hinder the free passage of macromolecules to the site of DNA synthesis, and it becomes difficult to evaluate the importance of cell components which may be necessary in this process. We have, therefore, searched for an alternate system which would contain the necessary components to carry out DNA synthesis and yet minimize the compartmentalization problems inherent in the use of cells or isolated nuclei. In this report, we describe a system which utilizes isolated chromatin for studying DNA synthesis in vitro. The system should be of use in evaluating the need for regulatory or accessory proteins in DNA synthesis and should permit analysis of DNA polymerase activity using "natural" templates.

Materials and Methods

[^{3}H]Deoxynucleoside triphosphates were purchased from Schwarz/Mann Biochemical Corp. and [^{3}H]- and [α-^{32}P]UTP were from the New England Nuclear Corp. Phenylmethanesulfonyl fluoride was obtained from Sigma Chemical Co. and Sarkosyl NL-97 from the CIBA-Geigy Corp.

Growth of Cells. HeLa S-3 and HeLa F cells (Flow Laboratories) were grown as described previously (Bolden et al., 1975). HeLa F cells were synchronized by the detachment method using a double thymidine block (Pederson and Robbins, 1971). Unless otherwise noted, all experiments presented in figures and tables were performed with extracts from randomly growing cells.

Preparation of Nuclei. Nuclei were prepared by procedures described by Berkowitz et al. (1969), Krokan et al. (1975a), or Yamashita and Green (1974) (RSB buffer was used with 2.5 mM EDTA). Any of these procedures may be used to prepare nuclei and yield chromatin of similar properties.

Chromatin Isolation. Nuclei (0.5 to 1 g) were suspended in 2 to 3 mL of buffer I (1 mM Tris[1]-HCl buffer, pH 8, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$), 1 mM EDTA) and centrifuged at 15 000g for 5 min at 0 °C. The pellet was resuspended and the nuclei were disrupted by gentle douncing with a loose fitting pestle in buffer II (buffer I without $PhCH_2SO_2F$) and again centrifuged at 15 000g, and the pellet was resuspended in buffer III (buffer I with 0.1 mM EDTA and 40% (v/v) glycerol). The washing was repeated a second time with buffer III omitting $PhCH_2SO_2F$ and the final chromatin preparation was suspended in 1 to 2 mL of the same buffer. Such chromatin preparations may be stored at −70 °C for several weeks without any loss of endogenous DNA synthesizing activity. Determination of the DNA and protein content of these chromatin preparations showed a ratio of 0.1 to 0.2 expressed in μg of DNA/μg of protein.

Assay for DNA Synthesis in Chromatin. The reaction mixture contained, in a final volume of 100 μL: 50 mM Tris-HCl buffer (pH 7.5), 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate (PEP), 8.5 units/mL pyruvate kinase (PK), 0.25 mM each of ATP, CTP, GTP, UTP, 0.1 mM each of dATP, dGTP, dCTP, and 0.05 mM [^{3}H]dTTP (specific activity 1000 cpm/pmol). Unless otherwise noted, the chromatin concentration expressed in DNA was 250 μg/mL. A chromatin content of 10 μg of DNA was assumed to be equivalent to 10^6 nuclei. The incubation was performed at 37 °C and aliquots of the reaction mixture were spotted onto Whatman GF/C glass filters and treated to determine acid-insoluble radioactivity as described by Bollum (1959).

Purification and Assay of DNA Polymerases α, β, and γ. DNA polymerases α, β, and γ were purified and assayed as

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; PEP, phosphoenolpyruvate; PK, pyruvate kinase.

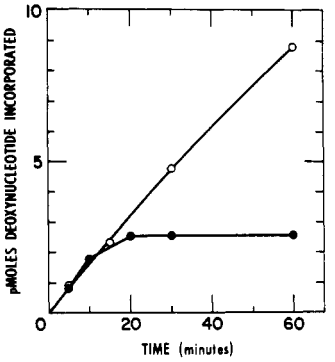

FIGURE 1: Kinetics of DNA synthesis in isolated nuclei and chromatin. HeLa S-3 chromatin was isolated as described in Materials and Methods. Nuclei from HeLa S-3 cells were isolated and assayed as described previously (Bolden et al., 1975). The amount of nuclei (●) and chromatin (○) in this experiment was 1×10^6 nuclei equivalents. The data are expressed as picomoles of total deoxynucleotide (dNTP) incorporated into an acid-insoluble form.

TABLE I: Requirements of the HeLa Cell Chromatin System for DNA Synthesis.[a]

Reaction mixture	Activity (%)
Complete	100.0
−dATP, dCTP, dGTP	2.9
+ DNase I, 5 μg	6.6
− phosphoenolpyruvate, pyruvate kinase	67.0
− ATP, phosphoenolpyruvate, pyruvate kinase	56.0
+ ATP, −(CTP, GTP, UTP)	100.0
+ ribonuclease A, 500 units; T_1 RNase, 250 units	100.0
+ EDTA, 10 mM	9.2
+ NEM, 2.5 mM	56.0

[a] Assays were performed for 30 min as described. The chromatin template concentration was about 250 μg/mL DNA and the complete incubation mixture is described in Materials and Methods. The complete system incorporated 12 pmol of deoxynucleotide into an acid-soluble form under these conditions.

described previously (Knopf et al., 1976). DNA polymerases α and β are hydroxylapatite fractions and the γ-polymerase was obtained from DNA-cellulose chromatography. Units were determined under optimal assay conditions with 250 μg/mL activated salmon sperm DNA as template for the α- and β-polymerases and 50 μg/mL of $(dT)_{12-18}$·poly(A) for the γ-polymerase assays.

Alkaline Sucrose Sedimentation. Alkaline sucrose sedimentation was carried out in 5–25% sucrose with a 60% sucrose cushion in a buffer containing 0.2 M NaOH, 1 M NaCl, 10 mM EDTA, 0.1% Sarkosyl NL-97. Before being placed on the gradients, aliquots of the reaction mixtures were treated with proteinase K (Stafford and Bieber, 1975) and then alcohol precipitated in the presence of 1 M LiCl. The dried precipitate was dissolved in 0.25 M NaOH and 0.1% Sarkosyl NL-97 and applied to the top of the gradients. Centrifugation was carried out in a Spinco SW 50.1 rotor at 35 000 rpm for 15 h at 20 °C. For comparison, a sonicated calf thymus DNA marker whose sedimentation coefficient was determined to be 4.1 S (under denaturing conditions) was run in parallel tubes.

Formaldehyde-CsCl-Cs_2SO_4 Density Gradient Centrifugation. The RNA and DNA products synthesized in the chromatin system were analyzed by the density gradient isopycnic centrifugation technique previously described (Spadari and Weissbach, 1975). After incubation, 0.5 mL of the chromatin reaction mixtures was treated with proteinase K (Stafford and Bieber, 1975), phenol extracted, and alcohol precipitated. The precipitate was dissolved in formaldehyde buffer and then denatured before addition to the gradient (Spadari and Weissbach, 1975).

DNA concentrations were determined by the method of Giles and Myers (1965). Protein concentrations were determined according to Böhlen et al. (1973) and Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Preparation of Chromatin for in Vitro DNA Synthesis. Chromatin from HeLa S-3 cells, prepared under the described conditions, showed endogenous DNA synthesis activity. We have compared the time course of the DNA synthesis in the chromatin preparation with that of an equivalent amount of nuclei. It was observed (Figure 1) that the DNA synthesis, as measured by [^{3}H]thymidine triphosphate incorporation, ceased after about 15 min in nuclei, whereas chromatin exhibited a linear synthesis of DNA for 60 min and will, in fact, continue for at least 2 h. The rate of synthesis of DNA was similar in both systems in the first 10 min and showed an incorporation of 2–10 pmol of deoxynucleoside triphosphates per 10^6 nuclei equivalents.

The requirements for the observed endogenous in vitro DNA synthesis with chromatin are outlined in Table I. In the absence of three deoxynucleotides, the DNA synthesis activity was drastically reduced. Addition of pancreatic DNase to the system also abolished the DNA synthesis; these data strongly suggested that the [^{3}H]thymidine triphosphate was incorporated into DNA. Synthesis was partially dependent on the presence of ATP and a triphosphate generating system since omission of pyruvate kinase and phosphoenolpyruvate, with or without ATP, inhibited the reaction about twofold. The presence or omission of GTP, CTP, or UTP had no effect and DNA synthesis was also unaffected by high concentrations of RNase A and T_1 RNase. Incubation of chromatin with *N*-ethylmaleimide resulted in a 50% inhibition of DNA synthesis. This result suggests that the β-polymerase might be the DNA polymerase activity responsible for the residual DNA synthesis since purified α-polymerase would be expected to be inhibited over 90% and γ-polymerase about 80% by *N*-ethylmaleimide under these conditions (Knopf et al., 1976; Matsukage et al., 1975). DNA β-polymerase is not noticeably inhibited by this level of *N*-ethylmaleimide (Knopf et al., 1976).

The influence of various salts on endogenous chromatin DNA synthesis was tested, and the experimental results are presented in Figure 2. Monovalent compounds such as NH_4Cl, NaCl, and KCl at 100 mM concentration stimulated the DNA synthesis about 1.5-, 1.7-, and 1.9-fold, respectively. The multivalent salts $[NH_4]_2SO_4$ and K_2HPO_4 stimulated DNA synthesis about twofold at a concentration of 50 mM.

Comparison of DNA Synthesis in Chromatin Prepared from Cells in the Mitotic or S Phase. The in vitro DNA synthesis in chromatin obtained from mitotic or S-phase HeLa F cells has been compared. Mitotic cells, or cells 4 h into the S phase, were prepared as described in Materials and Methods. The time course of DNA synthesis in these chromatin preparations is presented in Figure 3. S-phase chromatin exhibited about a five times higher rate of DNA synthesis than the mitotic chromatin. Thus it appears that the in vitro chromatin DNA synthesis reflects the actual cellular DNA synthesis occurring at the time of the chromatin preparation. The low level of DNA synthesis observed with mitotic chromatin preparations may reflect contamination with cells from dif-

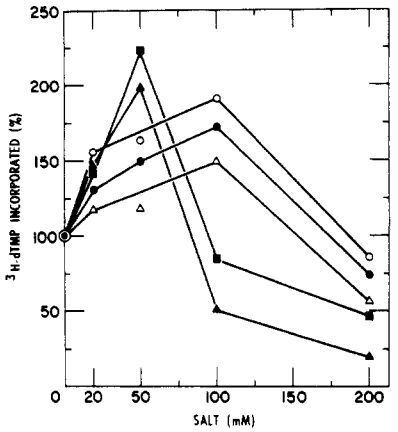

FIGURE 2: Effect of salt upon the [^{3}H]TMP incorporation of isolated chromatin. Assays were performed with HeLa S-3 chromatin (100 μg/mL DNA) for 30 min as described in presence of different concentrations of the following compounds: (●) NaCl; (○) KCl; (▲) $[NH_4]_2SO_4$; (△) NH_4Cl; and (■) KPO_4.

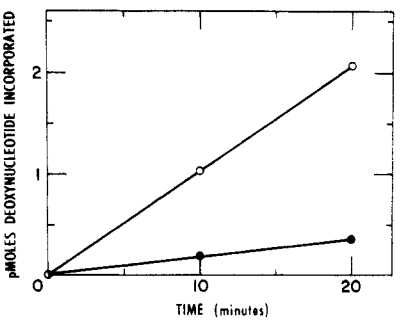

FIGURE 3: Kinetics of DNA synthesis of chromatin isolated from HeLa F cells in mitotic and S phase. Chromatin was isolated from mitotic and 4-h S-phase HeLa F cells as described in Materials and Methods. Incubations were carried out as described in legend of Figure 1 with a chromatin concentration of 1×10^6 nuclei equivalents.

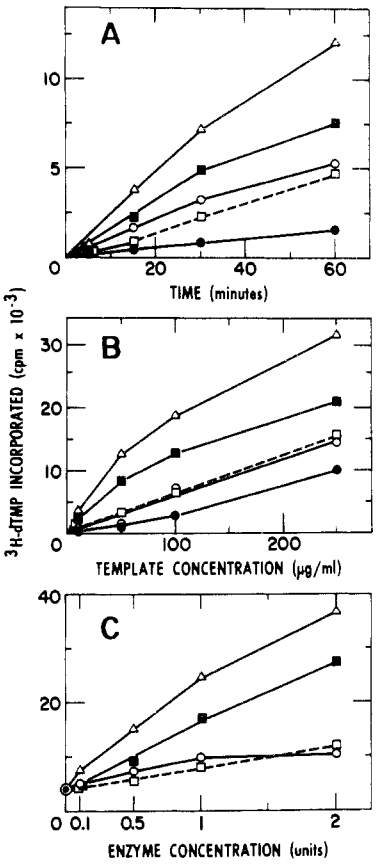

FIGURE 4: Chromosomal DNA synthesis in the presence and absence of added DNA polymerase α, β, and γ. (A) Time course of chromosomal DNA synthesis. Assays were performed with a chromatin concentration of 50 μg/mL DNA in absence (●) or in presence of 1 unit of each of the HeLa cell DNA polymerase α (○), β (■), γ (□) or a mixture of $\alpha + \beta + \gamma$ (△) as described. (B) Effect of the template concentration. Assays, enzyme concentrations, and symbols were as given in panel A. Incubations were for 30 min at 37 °C. (C) Effect of the enzyme concentration. Assays and symbols are as described in panel A. The chromatin concentration was 100 μg/mL DNA. Incubations were carried out for 20 min at 37 °C.

ferent stages or might be due to damage of the chromatin during the isolation procedure. Analogous results have been reported with isolated nuclei obtained from S-phase or nonsynchronized cell (Bolden et al., 1975; Bernard and Brent, 1973; Friedman, 1974), where it can be shown that nuclei from the S phase will synthesize DNA in vitro two to five times faster than nuclei from nonsynchronized growing cells.

Effect of Addition of DNA Polymerases. We have also asked whether chromatin can be utilized as a template by the three major mammalian DNA polymerases, α, β, and γ. In the first experiment (Figure 4A), the time course of DNA synthesis was analyzed in the absence or presence of exogenous DNA polymerase. In these experiments, equal activities of each enzyme (in units) were added to chromatin. The addition of α- or γ-polymerase resulted in a two- to threefold stimulation of the rate of DNA synthesis, whereas addition of β-polymerase gave a fivefold stimulation. Addition of all three DNA polymerases produced a rate of DNA synthesis which represented the additive value of each of the single enzymatic activities. When DNA synthesis was measured with constant amounts of the enzymes but increasing chromatin concentrations (Figure 4B), it was found that the increase in rate of DNA synthesis obtained with exogenously added DNA polymerases occurred at all template concentrations tested. Thus, the chromatin template was not saturating in this system since increasing rates of DNA synthesis occurred with a given enzyme concentration as the chromatin template concentration increased. The inverse experiment involving measurement of DNA synthesis at a constant chromatin concentration and varying enzyme concentrations (Figure 4C) again documented that addition of β-polymerase stimulated about six- to sevenfold. In all cases, the rates of DNA synthesis were found to be additive when all three polymerases were present in the reaction mixture.

Size Measurement of the DNA Products Synthesized by the Chromatin System in Absence and Presence of Exogenously Added DNA Polymerases. Analysis of the newly synthesized DNA products as well as the chromosomal DNA template, in the absence or presence of exogenously added DNA polymerases, was carried out by alkaline sucrose gradient velocity sedimentation (Figures 5A and 5B). The parental DNA template, which was labeled in vivo by a 10-min pulse with [^{3}H]thymidine, was found to sediment onto the 60% sucrose cushion and had therefore a sedimentation coefficient larger than 10 S. In fact previous reports have shown the cellular DNA is larger than 40 S when analyzed on alkaline sucrose gradients (Tseng and Goulian, 1975). The newly synthesized DNA, which was obtained by a 20-min in vitro incubation of chromatin in the presence of [α-^{32}P]dTTP, showed a heterogeneous sedimentation profile with much of the material sedimenting at about 4–6 S. Thirty-six percent of the newly synthesized DNA was found to sediment at greater than

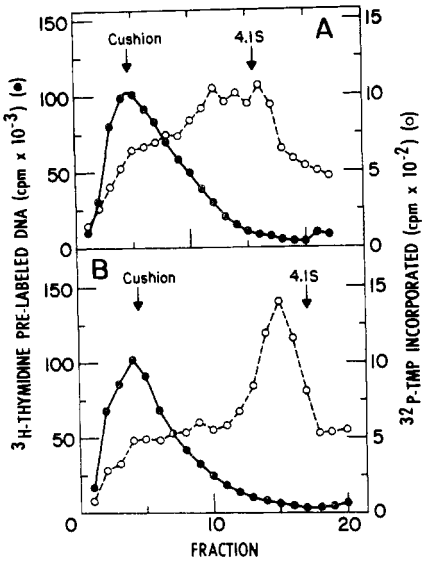

FIGURE 5: Size of the products of chromatin DNA synthesis in vitro. (A) Size of the products of the endogenous reaction. Chromatin was isolated from HeLa S3 cells which had been pulse labeled for 10 min with [^{3}H]-thymidine (20 μCi/mL) in vivo prior to the isolation of the chromatin. Assays (0.25 mL) were performed with 12.5 μM [α-^{32}P]TTP (specific activity 4800 cpm/pmol) and a chromatin template concentration of 500 μg/mL. Incubations were for 20 min at 37 °C as described. Sonicated calf thymus DNA, whose sedimentation coefficient was determined by alkaline band sedimentation in the analytical ultracentrifuge, was used as external marker. (B) Size of the products of the chromatin DNA synthesis in presence of added DNA polymerases. Assay conditions were used as described above, except that 0.5 unit of DNA polymerase α and β and 0.3 unit of DNA polymerase γ were added to the reaction mixture.

10 S (Figure 5A). Figure 5B shows the sedimentation of parental and newly synthesized DNA in presence of about 0.5 unit of the α- and β-polymerases and of about 0.3 unit of γ-polymerase. As before, a large fraction of the newly synthesized DNA was found in the 6S region of the sucrose gradient and about 33% at the position of the fast-sedimenting parental template DNA (fractions 1-10). These results may be compared with those of Krokan et al. (1975b) who found the synthesis of DNA fragments from 4 to 14 S occurring in isolated nuclei. These fragments could eventually be ligated to DNA pieces larger than 30 S.

Synthesis and Methylation of RNA. We have tested the endogenous chromatin system to find out if it was able to simultaneously synthesize RNA as well as DNA under the conditions used. RNA synthesis was initially assayed under conditions optimal for RNA polymerases using 20 mM ammonium sulfate (Schwartz et al., 1974), but we have also found that the optimal assay conditions for DNA synthesis in chromatin were also good for the incorporation of UMP into RNA. On the average, we observed twofold more RNA synthesis than DNA synthesis, i.e., in 10 min about 4-20 pmol of ribonucleotides were incorporated by 10^6 nuclei equivalents. Formaldehyde-CsCl-Cs_2SO_4 density gradient analysis showed that the [^{32}P]UMP-labeled product banded at the density of RNA and other experiments showed it to be completely degraded by RNase I (data not shown). The average size of the RNA products was determined to be approximately 8 S (Y. Furuichi, personal communication). The use of chromatin templates and RNA polymerase for transcriptional studies of genetic expression has already been reported (Axel et al., 1973). Further experiments were undertaken in order to show that methylation of nucleic acids also occurs in the chromatin system. In these experiments, chromatin incubations were carried out in presence of [^{3}H]methyl-*S*-adenosylmethionine. Approximately 1.3 pmol of [^{3}H]methyl groups were transferred to 10^6 nuclear equivalents of chromatin in 20 min under the conditions used (Table I). The labeled chromatin was then processed, as described in Materials and Methods, for CsCl-C_2SO_4 density gradient analysis. All of the [^{3}H]methyl label was in the region of the gradient where RNA bands and none was in the DNA region. RNA and DNA in eukaryotic cells normally contain methyl groups, and previous reports (Perry and Kelly, 1974; Roy and Weissbach, 1975) have demonstrated that methylation of RNA and DNA does occur in the nucleus.

Discussion

The results of this study indicate that chromatin preparations represent a useful tool for the study of DNA synthesis. In many ways, the chromatin synthesis mimics the characteristics of isolated, intact nuclei. The rate of DNA synthesis is roughly the same in vitro in nuclei and chromatin for about 10 min. After that, isolated nuclei show a marked slowing of synthesis whereas the chromatin DNA synthesis continues at a linear rate for at least 60 min. Nuclei and chromatin are also similar in that they reflect the DNA synthetic activity of the cells from which they are obtained. Thus, nuclei or chromatin derived from cells in S phase, which are actively making DNA, are several times more active than preparations derived from mitotic cells which are not synthesizing DNA. The requirements for DNA synthesis in chromatin, in vitro, are similar to those of isolated nuclei, although a more pronounced requirement for ATP is shown by the nuclear system (Bolden et al., 1975; Friedman, 1974). We have not investigated the effect of cytosol preparations on the ability of chromatin to synthesize DNA, though stimulation of isolated nuclei by cytoplasmic fractions has been reported (Friedman and Mueller, 1968).

The size of the single-stranded DNA segments made in isolated chromatin after 20 min is about 4-6 S (65% of the product) with about another 35% showing a larger size. These lengths also resemble the size of the DNA pieces made in isolated nuclei in the absence of cytoplasmic factors (Friedman and Mueller, 1968; Bernard and Brent, 1973; Friedman, 1974). Isolated nuclei are known to be able to synthesize RNA (Berkowitz et al., 1969) in vitro and available evidences suggest that both DNA and RNA methylation occurs in the nucleus of the cell (Perry and Kelly, 1974). Our chromatin preparations can also synthesize RNA as well or faster than DNA is synthesized and methylation of RNA also takes place in this in vitro system.

There is at least one important advantage the chromatin system may offer workers interested in the enzymology of DNA replication. We have shown that chromatin preparations respond to the presence of exogenous homologous DNA polymerases such that DNA synthesis in chromatin can be stimulated two- to sixfold by addition of DNA polymerases α, β, and γ. In contrast, we have found no response or a variable, minor stimulation when DNA polymerases are added to isolated nuclei. The possibility of gaining more insight into the role of the DNA polymerases by the use of chromatin templates is now under study. The use of chromatin as a "natural" template, which resembles the in vivo DNA replication system, may provide a valuable tool for studying other components of the replication system in addition to the DNA polymerases.

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Partial Purification and Characterization of a Uracil DNA *N*-Glycosidase from *Bacillus subtilis*†

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ABSTRACT: A uracil specific DNA *N*-glycosidase activity has been partially purified from crude extracts of *Bacillus subtilis*. The enzyme has a molecular weight of approximately 24 000 with no subunit structure. It has no requirement for any known cofactors but is inhibited in the presence of Co^{2+}, Fe^{2+}, or Zn^{2+}. The enzyme is specific for uracil in single- and double-stranded deoxyribonucleopolymers and does not release free uracil from RNA or from poly(rU):poly(dA). In addition, neither Udr, dUMP, nor dUTP is recognized as substrate. The enzyme will attack small poly(dU) oligomers but the minimum size recognized as substrate is $(pU)_4$. This enzyme may have a role in the repair (by base excision) of uracil in DNA arising either by incorporation during DNA synthesis or by deamination of cytosine in DNA.

In recent years an enzyme activity has been identified in both prokaryote and eukaryote sources that catalyzes the removal of uracil from DNA as the free base (Carrier and Setlow, 1974; Lindahl, 1974; Friedberg et al., 1975; Lindahl, 1975; Duncan et al., 1976a; Katz et al., 1976). In previous studies from this laboratory (Friedberg et al., 1975; Duncan et al., 1976a), we showed that this activity (referred to as uracil DNA *N*-glycosidase) is present in extracts of uninfected *B. subtilis*. We also showed that, following infection of *B. subtilis* by phage PBS2 (which contains uracil instead of thymine in its DNA), the *N*-glycosidase activity is no longer present in crude extracts. This phenomenon is dependent on protein synthesis after phage infection, suggesting that the phage codes for an inhibitor of the *N*-glycosidase.

We have purified both the host uracil DNA *N*-glycosidase activity and the phage induced inhibitor and are studying their properties and interaction. In the present report we describe the purification and characterization of the enzyme. Studies on the inhibitor will be presented later.

Materials and Methods

1. Cells and Cell Growth. Strain SB 168 (obtained from Dr. A. T. Ganesan, Genetics Department, Stanford University) was grown in double strength nutrient broth (Difco) or in brain heart infusion broth (Difco) at 37 °C. The cells were harvested in late log or stationary phase and stored at −20 °C.

2. Enzyme Purification. (a) Crude Extract. Frozen cells

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