Characterization of DNA Synthesis in Salt-Treated HeLa Cell Nuclei

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ABSTRACT. The effects of salt treatment of isolated nuclei on the nature of DNA synthesis and on the solubilization of DNA polymerase α have been investigated. Treatment with 0.2 M KCl resulted in a deficiency in the function to join Okazaki fragments, whereas the synthesis of these fragments was little affected. No DNA synthesis was observed in 0.3 M KCl-treated nuclei. DNA synthesis in 0.2 M KCl-treated nuclei required ATP, Mg^{2+} and four deoxyribonucleoside triphosphates, and was markedly inhibited by arabinofuranosylcytosine triphosphate and aphidicolin, but not by 2',3'-dideoxythymidine triphosphate. This suggests that the DNA synthesis is the replicative-type, catalyzed by DNA polymerase α . DNA polymerase α in 0.3 M KCl-nuclear extract was detected as two active forms distinct in their binding affinities for DNA. In 0.2 M KCl-treated nuclei, only one active form with high binding affinity was retained, indicating that this form is responsible for the synthesis of Okazaki fragments.

The mechanism of DNA replication in mammalian cells is complex, and its details have yet to be clarified. The identification and characterization of replication factors in connection with the processes of DNA replication are an important step toward determining the molecular mechanism of DNA replication.

Isolated nuclei from mammalian cells have proved a useful experimental system for the study of DNA replication *in vitro* (1-10). Although by using such an *in vitro* system, DNA replication of mammalian cells has been shown to proceed in a discontinuous manner through the formation of short fragments (Okazaki fragments) which are subsequently converted into high molecular weight DNA, the nuclear system is too complex to study the factors involved in the processes of DNA replication.

Seki and Mueller (11) have demonstrated the dissociation and reconstitution of the nuclear system by mild salt treatment. Such a reconstituted subnuclear system using salt-treated nuclei as a "natural template" may be valuable for identifying and characterizing the factors involved in DNA replication. Recently, by using a reconstituted subnuclear system, Brun and Weissbach (12) found evidence that suggests that RNA polymerase I is involved in the priming event for DNA synthesis.

In the course of our study on the effect of salts on DNA synthesis in isolated nuclei, we observed that in the presence of 0.2 M KCl, isolated nuclei can synthesize Okazaki fragments, but are deficient in the function to join them. Thus, it seemed possible that by treatment of isolated nuclei with salts at appropriate concentrations, factors

necessary for some step of DNA replication could be selectively solubilized from nuclei, leaving salt-treated nuclei capable of other steps of DNA replication.

We have analyzed here the nature of DNA synthesis in HeLa cell nuclei after treatment with varying concentrations of KCl. In addition, we have characterized the nuclear salt-extract in regard to DNA polymerase α , a candidate for a replication enzyme (13–20). We provide evidence that isolated nuclei contain two active forms of DNA polymerase α distinct in their binding affinities for DNA, and that one active form retained in 0.2 M KCl-treated nuclei is responsible for the synthesis of Okazaki fragments.

MATERIALS AND METHODS

Isolation and salt treatment of nuclei. Nuclei were isolated from HeLa S3 cells 3 h after release from the hydroxyurea block as described previously (21, 22). Salt treatment of isolated nuclei was performed according to the method of Seki and Mueller (11). Isolated nuclei were suspended in 9 volumes of buffer A (10 mM Tris-HCl (pH 7.8), 3 mM MgCl₂, 1 mM Na₂EDTA, 2 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonylfluoride) containing various concentrations of KCl. After being kept at 0°C for 20 min, the suspension was centrifuged at $800 \times g$ for 5 min. The precipitate was washed once with buffer A then centrifuged as before to pellet the salt-treated nuclei.

Assay for DNA synthesis. The activity of DNA synthesis in isolated and salt-treated nuclei was assayed in a standard reaction mixture containing 100 mM KCl, 40 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 1 mM Na₂EDTA, 2 mM 2-mercaptoethanol, 4 mM ATP, 100 μ M each dATP, dGTP, dCTP, and 20 μ M [³H]dTTP (0.67 Ci/mmol). The concentration of nuclei was set at 1×10^6 /tube (total volume of 150 μ l). The radioactivity incorporated into 5% trichloroacetic acid precipitable material was collected on a Whatman GF/C glass fiber filter and counted with a liquid scintillation spectrometer (21, 22).

Alkaline sucrose gradient. DNA was pulse-labelled by incubating 1×10^6 nuclei at 37°C in the standard reaction mixture, except that the concentration of [³H]dTTP was 10 μ M (3.4 Ci/mmol). Nuclei were lysed by an addition of alkaline lysis solution containing 0.3 M KOH, 0.7 M KCl, 10 mM Na₂EDTA and 2% sarkosyl. The lysate was kept at 4°C for at least 2 h before applying it to an alkaline sucrose gradient. The alkaline sucrose gradient was 5–20% sucrose in a solution containing 0.3 M KOH, 0.7 M KCl, 1 mM Na₂DETA and 0.1% sarkosyl (8, 9). Centrifugation was performed at 22,500 r.p.m. for 14 h at 4°C in a Hitachi RPS-25 rotor.

Assay for DNA polymerase activity. DNA polymerase α , β and γ were assayed essentially according to the method described by Spadari *et al.* (15).

α assay: The 150 μ l assay mixture contained 50 mM KCl, 40 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 1 mM Na₂EDTA, 2 mM 2-mercaptoethanol, 100 μ M each dATP, dGTP and dCTP, 20 μ M [³H]dTTP (0.67 Ci/mmol) and 45 μ g activated DNA. Samples were incubated at 37°C for 30 min. The reaction was stopped by chilling the mixture to 0°C and by the addition of carrier DNA to a final concentration of 200 μ g/ml and trichloroacetic acid at 5%. The DNA precipitate was collected and its radioactivity was measured as described above.

β assay: The assay was essentially the same as that for polymerase α , except that 10 mM *N*-ethylmaleimide was present.

γ assay: The 150 μ l assay mixture contained 100 mM KCl, 40 mM Tris-HCl (pH 7.8), 0.5 mM MnCl₂, 2 mM 2-mercaptoethanol, 40 μ M [³H]dTTP (0.33 Ci/mmol) and 5 μ g

poly(rA)-oligo(dT)₁₂. Incubation was carried out at 37°C for 30 min. The amount of incorporated [³H]dTTP into the homopolymer was measured as described under the polymerase α assay.

Single stranded-DNA cellulose column chromatography. Single stranded-DNA cellulose was prepared essentially according to the method of Alberts and Herrick (23) with slight modification. The DNA-cellulose mixture was dried at room temperature by evacuation in a dessicator containing P₂O₅ instead of by lyophilization. This procedure yielded DNA-cellulose with a higher concentration of ligand than with the original method. The nuclear salt-extract was centrifuged at 105,000 $\times$ g for 1 h then dialyzed against buffer B (20 mM Tris-HCl (pH 8.1), 1 mM Na₂EDTA, 2 mM 2-mercaptoethanol and 10% glycerol) containing 50 mM KCl. All operations were carried out at 0–4°C. The sample was applied to a column previously equilibrated with 50 mM KCl-buffer B, and was washed with the same buffer then eluted stepwise with buffer B containing 0.18, 0.27 and 0.55 M KCl.

RESULTS AND DISCUSSION

Effect of KCl on DNA synthesis in isolated nuclei. We first examined the effect of salt on DNA synthesis in isolated nuclei. DNA synthesis was critically dependent on the concentration of KCl in the reaction mixture (Fig. 1). This result agrees with the observation of Hershey *et al.* (6). Maximal activity was observed at 0.1 M KCl. The overall activity of DNA synthesis in isolated nuclei at 0.04 and 0.2 M KCl was reduced by about one half that at the optimum.

Products synthesized at the three different ionic strengths, 0.04, 0.1 and 0.2 M KCl, were analyzed by sedimentation in alkaline sucrose gradients. As shown in Fig. 2, about 50% of the product synthesized at 0.1 M KCl after a 10-min incubation was distributed in short fragments, the rest was in high molecular weight DNA fractions toward the bottom. At 0.04 M KCl, both the syntheses of short fragments and high molecular weight DNA decreased to about 50% of the values at 0.1 M KCl. The product synthesized at 0.2 M KCl was predominantly in short fragments with a slight decrease in the amount as compared to 0.1 M KCl. At this concentration, little radioactivity was detected in the high molecular weight DNA fractions after additional incubation. The deficiency in the function to synthesize high molecular weight DNA in isolated nuclei in the presence of 0.2 M KCl may be due to the dissociation of the factors necessary for joining the short fragments. This possibility is confirmed in the next section.

The decrease in DNA synthesis at 0.04 M KCl may be due to the reduction of DNA chain growth rate within the short fragments (Okazaki fragments). Nuclei pre-treated with 0.2 M KCl (0.2 M KCl-treated nuclei), which synthesize Okazaki fragments only, as described in the following section (Fig. 4), are suitable to examine this possibility. The pattern of the dependency curve of KCl in the 0.2 M KCl-treated nuclei was similar to that of isolated nuclei (Fig. 1). 0.1 M KCl gave maximal activity. The product analysis by sedimentation in an alkaline sucrose gradient revealed that both the amount and size of the Okazaki fragments synthesized at 0.04 M KCl were smaller than those synthesized at 0.1 M KCl (Fig. 3). At the three different ionic strengths with the omission of ATP, the DNA synthesizing activities were less than 10% of the optimum, and the products were found in fragments smaller than the Okazaki fragments.

These results suggest that the ionic environment has a critical effect on the opera-

Fig. 1. Effect of KCl on DNA synthesis in isolated and 0.2 M KCl-treated nuclei. Activities of DNA synthesis in isolated (—●—) and in 0.2 M KCl-treated (—○—) nuclei were assayed in the standard reaction mixture except for the concentration of KCl. Incubation was carried out for 10 min at 37°C. The incorporation of [³H]dTMP into acid insoluble material was measured as described under MATERIALS AND METHODS.

Fig. 2. Sedimentation of newly synthesized DNA in isolated nuclei in an alkaline sucrose gradient at different concentrations of KCl. Isolated nuclei were incubated for 10 min at 37°C in the standard reaction mixture except for the concentration of KCl; 0.04 (—▲—), 0.1 (—●—) and 0.2 (—■—) M. The products were lysed by adding alkaline lysis solution, then they were centrifuged in alkaline sucrose gradients as described under MATERIALS AND METHODS. The arrow indicates the location of ¹⁴C-labelled DNA of the nucleosome monomer from HeLa S3 cells (4-5S). The top of the gradient is at the right, the direction of sedimentation is to the left.

Fig. 3. Analysis of the size distribution of the DNA synthesized in 0.2 M KCl-treated nuclei at different concentration of KCl. 0.2 M KCl-treated nuclei were incubated for 10 min at 37°C in the standard reaction mixture except for the concentration of KCl; 0.04 (—▲—) and 0.1 (—●—) M. The same marker as in Fig. 2 was used. The top of the gradient is the right, the direction of sedimentation is to the left.

tion of the DNA replication apparatus in isolated nuclei by affecting the interactions of the replication factors with DNA and/or each other.

DNA synthesis in salt-treated nuclei. Treatment of isolated nuclei with appropriate concentration of salt, may make it possible to solubilize selectively the replication factors required for some step of DNA replication, leaving the nuclei capable of carrying out another step of DNA replication. To test this possibility, we treated isolated nuclei with KCl at concentrations higher than 0.1 M and analyzed the products synthesized in salt-treated nuclei.

The nuclear structure of salt-treated nuclei was maintained intact even after KCl treatment at a concentration as high as 0.3 M. The optimal conditions and requirements for DNA synthesis in salt-treated nuclear systems were similar to those in the isolated nuclear system. As shown in Fig. 4A, the product synthesized in isolated nuclei, after a 10-min incubation, was distributed in short fragments and in high molecular weight DNA fractions as described above. In the 0.15 M KCl-treated

Fig. 4. Sedimentation in an alkaline sucrose gradient of newly synthesized DNA in isolated and 0.2 M KCl-treated nuclei. (A): isolated (—●—), and 0.15 (—○—), 0.2 (—■—), 0.25 (—□—) and 0.3 (—▲—) M KCl-treated nuclei were incubated for 10 min at 37°C. (B): isolated (—●—), 0.2 (—■—) and 0.3 (—▲—) M KCl-treated nuclei were incubated for 30 min at 37°C. The same marker as in Fig. 2 was used. The top of the gradient is at the right, the direction of sedimentation is to the left.

nuclei, short fragments accumulated, and high molecular weight DNA was synthesized with low efficiency. In the 0.2 M KCl-treated nuclei, short fragments were synthesized with slightly lower efficiency than in isolated nuclei; no significant amount of radioactivity was found in addition to that found in them. Low and little capacity to synthesize short fragments were observed in the 0.25 and 0.3 M KCl-treated nuclei, respectively. Although a marked increase in the synthesis of high molecular weight DNA was observed in isolated nuclei after a 30-min incubation as compared to 10-min, little radioactivity was found in the high molecular weight DNA fractions in the 0.2 M and 0.3 M KCl-treated nuclei even after the same duration of pulse length (Fig. 4B).

The short fragments observed in 0.2 M KCl-treated nuclei may correspond to Okazaki fragment-like DNA intermediates that should be joined into high molecular weight DNA, not to the artificial fragments produced by the action of endonucleases and/or uracil-DNA-*N*-glycosidase. This hypothesis is supported by the following observations. First, when 0.2 M KCl-treated nuclei were prepared from cells previously labelled with [^{14}C]thymidine, and when the size of the DNA was analyzed by sedimentation in an alkaline sucrose gradient, the size distribution was virtually identical with that of isolated nuclei (Fig. 5). Second, the joining function was re-

Fig. 5. Analysis of the size distribution of DNA in isolated and 0.2 M KCl-treated nuclei. Isolated and 0.2 M KCl-treated nuclei were prepared from cells previously labelled with [^{14}C]thymidine then incubated for 10 min at 37°C in the standard reaction mixture except that [^3H]dTTP was replaced by unlabelled dTTP. The samples first were lysed by the alkaline lysis solution then layered on alkaline sucrose gradients as described under MATERIALS AND METHODS. Sedimentation was carried out at 22,500 r.p.m. for 4 h at 4°C in a Hitachi RPS-25 rotor; isolated (—●—) and 0.2 M KCl-treated nuclei (—○—).

Fig. 6. Alkaline sucrose gradients demonstrating the effect of the complementary nuclear salt-extract on the size of DNA synthesized in 0.2 M KCl-treated nuclei. After 0.2 M KCl-treated nuclei had been pulse-labelled for 2 min at 37°C, 130-fold unlabelled dTTP and 3 nuclear equivalents of complementary salt-extract were added to the reaction mixture. The samples then were incubated for an additional 10 min after which they were prepared for alkaline sucrose sedimentation as described under MATERIALS AND METHODS; 0.2 M KCl-treated nuclei (—○—) and reconstituted nuclei (—●—).

stored to 0.2 M KCl-treated nuclei by again adding the complementary nuclear salt-extract (Fig. 6).

These results indicate that the short fragments synthesized in 0.2 M KCl-treated nuclei are Okazaki fragments, and that 0.2 M KCl-treated nuclei retain the replication factors and structure needed for the synthesis of Okazaki fragments, but that they are deficient in some essential factors for the joining of these fragments. The 0.2 M KCl-treatment of isolated nuclei, therefore, make it possible to dissect the replication factors connected with two steps of DNA replication; the synthesis and joining of Okazaki fragments.

Characterization of DNA synthesis in 0.2 M KCl-treated nuclei. To characterize DNA synthesis in 0.2 M KCl-treated nuclei, the requirements and the effect of DNA polymerase-inhibitors were investigated (Table 1). All four deoxyribonucleoside triphosphates were required for maximal activity. A marked reduction in activity was observed when even one deoxyribonucleoside triphosphate was omitted. The optimal concentration of ATP was around 4 mM. With the omission of ATP, the activity was less than 10% of the optimum. At an ATP concentration of 4 mM, the optimal Mg^{2+} concentration was 5 mM. With the omission of Mg^{2+} , activity was 5% of the optimum.

Arabinofuranosylcytosine triphosphate (araCTP) inhibits DNA polymerase α more strongly than β (24, 25). As shown in Table 1, a 5- or 10-fold molar excess of araCTP over dCTP caused more than 90% inhibition. 2', 3'-dideoxythymidine triphosphate (d_2TTP), a potent inhibitor of DNA polymerase γ and β (19, 25), essentially had no effect at a $d_2TTP/dTTP$ concentration ratio of 1.5 or 3. Aphidicolin, a specific inhibitor of DNA polymerase α (26, 27), completely inhibited DNA synthesis at a concentration of 1 or 3 $\mu g/ml$. These observations were corroborated by the identification of DNA polymerase in 0.2 M KCl-treated nuclei as described in the following section.

Our results strongly suggest that DNA synthesis in 0.2 M KCl-treated nuclei is of the replicative type, catalyzed by DNA polymerase α .

TABLE 1. REQUIREMENTS FOR AND THE EFFECT OF INHIBITORS ON DNA SYNTHESIS IN 0.2 M KCl-TREATED NUCLEI

Reaction mixture	% of control
Complete	100
Deletions	
–dATP	23
–dGTP	22
–dCTP	22
–dATP, dGTP, dCTP	4
– Mg^{2+}	5
–ATP	8
Additions	
araCTP 0.5 (mM)	7
1.0 (mM)	4
d_2TTP 30 (mM)	87
60 (mM)	79
Aphidicolin 1 ($\mu g/ml$)	4
3 ($\mu g/ml$)	1

The assays for DNA synthesis in 0.2 M KCl-treated nuclei were performed as described under MATERIALS AND METHODS with the deletions and additions listed above.

Identification of DNA polymerase α in 0.2 M KCl-treated nuclei. To identify the DNA polymerase responsible for the synthesis of Okazaki fragments, we analyzed two nuclear salt-extracts by chromatography of single stranded-DNA cellulose. One is the complementary nuclear extracts of 0.3 M KCl-treated nuclei (0.3 M KCl nuclear extract, NE-0.3) which is incapable of DNA synthesis by itself. Another is the extract from 0.2 M KCl-treated nuclei with 0.3 M KCl (0.2–0.3 M KCl nuclear extract, NE-0.2–0.3), which contains some essential factors for the synthesis of Okazaki fragments. Almost all the DNA polymerase activities in both nuclear extracts were inhibited by 10 mM *N*-ethylmaleimide. Low but appreciable amounts of DNA polymerase activity to utilize poly(rA)-oligo (dT)₁₂ as a template-primer were detected in both nuclear extracts. More than 95% of the nuclear DNA polymerase activity, sensitive to 10 mM *N*-ethylmaleimide, was extracted to NE-0.3.

Each nuclear extract was dialyzed against 0.05 M KCl-buffer B then applied to a single stranded-DNA cellulose column previously equilibrated with the same buffer. The column was washed with the same buffer then eluted stepwisely with buffer B containing 0.18, 0.27 and 0.55 M KCl. As shown in Fig. 7A, in the case of NE-0.3,

Fig. 7. Single stranded-DNA cellulose column chromatography. (A) 0.3 and (B) 0.2–0.3 M KCl nuclear extracts were prepared as described under MATERIALS AND METHODS. Each nuclear extract was applied to a single stranded-DNA cellulose column previously equilibrated with buffer B-0.05 M KCl, then washed the same buffer and eluted stepwisely with buffer B-0.18, 0.27 and 0.55 M KCl. Fractions were assayed for DNA polymerase α (—●—) and β (—△—).

two peaks of DNA polymerase α activity were detected. This heterogeneous chromatographic behavior of DNA polymerase α was reproducible. Bieri-Bonniot and Schuerch (28) have reported a similar result with poly(dT)-CL-Sepharose. Whereas with NE-0.2–0.3, one activity was eluted after changing the KCl concentration to 0.27 M (Fig. 7B). Low but appreciable amounts of DNA polymerase β activity, insensitive to 10 mM *N*-ethylmaleimide, were detected in 0.55 M KCl-eluted fractions. In both cases, the activities eluted at a KCl concentration lower than 0.55 M were inhibited by more than 95% by *N*-ethylmaleimide. The further purified, two active forms of DNA polymerase α differed in chromatographic behavior; in their salt requirements for optimal activity and in their K_m values for dTTP, although the two forms both exhibited properties characteristic of DNA polymerase α , sensitivity to *N*-ethylmaleimide and similar molecular size in velocity sedimentation (not shown).

These observations indicate that isolated nuclei contain two active forms of DNA polymerase α distinct in their binding affinities for DNA or chromatin, and that one active form with the higher binding affinity (retained in 0.2 M KCl-treated nuclei) is responsible for the synthesis of Okazaki fragments. These results are also supported by our observation (38) that the activity of the one form with higher binding affinity changed with a very close temporal relationship to *in vivo* DNA synthesis.

The widely reported heterogeneity of DNA polymerase α (29–36) may be correlated with its biological functions in the processes of DNA replication. More recently, Wist and Prydz (37) have demonstrated that DNA polymerase α is responsible for gapfilling as well as for elongation in discontinuous DNA synthesis in isolated nuclei. There is also the possibility that another form of DNA polymerase α with a lower binding affinity, may be involved in the filling process in the gaps between adjacent Okazaki fragments.

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