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Characterization of Deoxyribonucleic Acid Synthesis in Reconstituted Nuclear Systems†

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ABSTRACT: Reconstituted nuclear systems for the analysis of mammalian cell DNA replication were characterized in detail. These in vitro systems were made by adding back nuclear salt extracts to salt-treated nuclei which were deficient in some steps of DNA replication. In the reconstituted nuclear system consisting of 0.2 M KCl treated nuclei, which were deficient in the function to synthesize high molecular weight DNA, and their complementary nuclear extract, the function to convert Okazaki fragments to high molecular weight DNA was restored. The reconstitution between 0.3 M KCl treated nuclei, which had little capacity to synthesize DNA, and 0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei resulted in the restoration of the ability to synthesize Okazaki fragments. DNA synthesis in these reconstituted nuclear systems required ATP, magnesium ion, and four deoxyribonucleoside triphosphates and was markedly inhibited by aphidicolin and by 1-β-D-arabinofuranosylcytosine 5′-triphosphate but not by 2′,3′-dideoxythymidine triphosphate. By use of 0.2 M KCl treated nuclei and a reconstituted nuclear system consisting of 0.2 M KCl treated nuclei and their complementary nuclear extract, the requirement of ATP in the processes of DNA replication was investigated. A high level of ATP was required for the synthesis of Okazaki fragments, and it was substituted by a high level of dATP and ADP completely and partially, respectively. The Okazaki fragments synthesized in 0.2 M KCl treated nuclei in the presence of a high level of ADP were converted to high molecular weight DNA with a low level of ATP in the presence of the complementary nuclear extract, whereas in the presence of a high level of dATP, the conversion was not observed even in the presence of a low level of ATP and the complementary nuclear extract.

Biochemical studies of DNA replication of mammalian cells have been difficult not only due to the complex nature of the replicative processes but also due to the absence of suitable systems for detecting enzymes and factors involved in the processes. The establishment of systems, which enable us to identify enzymes and factors and to evaluate their roles in connection with the processes of DNA replication, is an important step toward the elucidation of the molecular mechanism of mammalian DNA replication.

Although isolated nuclei from mammalian cells have been proved a useful experimental system for the study of the mechanism of DNA replication (Friedman & Mueller, 1968; Lynch et al., 1970; Hershey et al., 1973; Krokan et al., 1975a,b; Benbow & Ford, 1976; Jazwinski et al., 1976; Hooton & Hoffbrand, 1977; Seale, 1977; Wist, 1979; Oguro et al., 1980; Nagata et al., 1981), the nuclear system is too complex to study the factors involved in the processes of DNA replication. Seki & Mueller (1976) have demonstrated the dissociation and reconstitution of a nuclear system by mild salt treatment. By using a similar system, Brun & Weissbach (1978) provided the evidence suggesting that RNA polymerase I was involved in the priming event for DNA synthesis.

We have shown (Tanuma et al., 1980) that by mild salt treatment of isolated nuclei at appropriate concentrations, factors necessary for some steps of DNA replication can be selectively solubilized from isolated nuclei, leaving salt-treated nuclei capable of carrying out other steps of DNA replication. Therefore, the reconstituted nuclear system using the salt-treated nuclei as a "natural template" may become a valuable tool for the identification and subsequent characterization of factors involved in DNA replication.

We describe here the characterization of reconstituted nuclear systems made by adding back nuclear salt extracts to salt-treated nuclei. In addition, by using salt-treated nuclei and a reconstituted nuclear system, we have investigated the roles of ATP in the processes of the synthesis and subsequent joining of Okazaki fragments.

Materials and Methods

Materials. Aphidicolin was kindly supplied by Dr. S. Ikegami (University of Tokyo) and Dr. M. Ohashi (Tokyo Metropolitan Institute of Gerontology).

Cell Culture and Synchronization. HeLa S3 cells were maintained in a monolayer culture in Eagle's minimal essential medium supplemented with 10% calf serum. Synchronization was carried out by exposing cells to 1 mM hydroxyurea for 16 h.

Isolation of Nuclei. Nuclei were isolated from HeLa S3 cells 3 h after the release from the hydroxyurea block as described previously (Tanuma et al., 1979). Cells were suspended at a concentration of 2×10^7/mL in buffer A (10 mM Tris-HCl,[1] pH 7.8, 3 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM

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[1] Abbreviations: araCTP, 1-β-D-arabinofuranosylcytosine triphosphate; ddTTP, 2′,3′-dideoxythymidine triphosphate; EDTA, ethylendiaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride) containing 0.025% Triton X-100 and kept to be swollen for 7 min at 0 °C. The cell suspension was homogenized with 20 strokes in a Potter–Elvehjem type Teflon–glass homogenizer. The homogenate was centrifuged at 800*g* for 5 min to pellet nuclei. The nuclei were washed once with buffer A, and washed nuclei were resuspended in buffer A at a concentration of 4×10^7/mL.

Salt Treatment of Nuclei and Preparation of Nuclear Extracts. Isolated nuclei prepared as described above were suspended at a concentration of 8×10^7/mL in buffer A containing various concentrations of KCl. After keeping for 20 min at 0 °C, the suspension was centrifuged at 800*g* for 5 min. The supernatant and the pellet were taken as a nuclear extract and salt-treated nuclei, respectively. The nuclear extract was further centrifuged at 10000*g* for 10 min. The salt-treated nuclei were washed once with buffer A and suspended at a concentration of 4×10^7/mL in buffer A. NE-0.3~0.2 (0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei) was prepared as described above except that 0.2 M KCl treated nuclei were used instead of isolated nuclei and that the salt-treated nuclei were precipitated by centrifugation at 2200*g* for 5 min.

Assay for DNA Synthesis. The activity of DNA synthesis in isolated nuclei, salt-treated nuclei, and reconstituted nuclear systems was assayed in a reaction mixture containing 100 mM KCl, 40 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM 2-mercaptoethanol, 4 mM ATP, 100 μM each of dATP, dCTP, and dGTP, and 20 μM [^{3}H]dTTP (0.67 Ci/mmol). The concentration of nuclei was set at 1×10^6/tube (total volume 150 μL). Incubation was carried out at 37 °C for indicated times. The reaction was stopped by chilling, and 0.35 mL of cold distilled water and 0.5 mL of cold 10% trichloroacetic acid (final concentration 5%) were added to the reaction mixture successively. Trichloroacetic acid (5%) precipitable materials were collected on a Whatman GF/C glass fiber filter and washed 5 times with 2 mL of 5% trichloroacetic acid and once with 2 mL each of ethanol and acetone successively. The radioactivity was measured with a liquid scintillation spectrometer.

Alkaline Sucrose Gradient Centrifugation. DNA was labeled by incubating 1×10^6 nuclei at 37 °C in the standard reaction mixture except that the concentration of [^{3}H]dTTP was 10 μM (3.4 Ci/mmol). The nuclei was lysed by an addition of alkaline lysis solution containing 0.3 M KOH, 0.7 M KCl, 10 mM EDTA, and 2% sarkosyl. The lysate was kept at 4 °C for at least 2 h before it was applied to an alkaline sucrose gradient. The alkaline sucrose gradient was 5–20% sucrose in the solution containing 0.3 M KOH, 0.7 M KCl, 1 mM EDTA, and 0.1% sarkosyl. Centrifugation was performed at 52500*g* for 14 h in a Hitachi RPS 25 rotor.

Results

Properties of Salt-Treated Nuclear Systems. Successful dissociation and reconstitution of the HeLa nuclear system were attained only when nuclei were isolated with a hypotonic buffer as described under Materials and Methods. A divalent cation (3 mM $MgCl_2$) decreased the aggregation of nuclei and stabilized the integrity of nuclei as judged by microscopic observation and ability to synthesize DNA after keeping at 0 °C for up to 60 min. Contamination with cytoplasmic materials adherent to nuclei was decreased by adding as little as 0.025% Triton X-100 in the hypotonic buffer. Nuclei isolated under the above conditions maintained their nuclear structure even after KCl treatment at a concentration as high as 0.3 M.

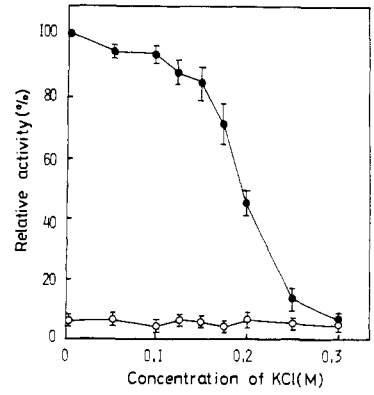

FIGURE 1: DNA synthesizing activity in salt-treated nuclei. Isolated nuclei were treated with various concentrations of KCl for 20 min at 0 °C as described under Materials and Methods. DNA synthesizing activity in the salt-treated nuclei was assayed in the standard reaction mixture with (●) or without (○) ATP for 10 min at 37 °C. Values are normalized with respect to the activity in isolated nuclei (100%). 100% corresponds to 6.3 pmol of dTMP incorporation. Vertical lines indicate standard deviation.

Figure 1 shows the DNA synthesizing activity in salt-treated nuclei after treatment with various concentrations of KCl for 20 min at 0 °C. The decrease of ATP-dependent activity was not impressive at KCl concentrations below 0.15 M. Above 0.15 M, however, a marked decrease in the activity was observed. At 0.2 and 0.3 M KCl, the activity was reduced to less than 50% and 10% of that in isolated nuclei, respectively. The result is in accord with the observation of Seki & Mueller (1976). The optimal conditions and requirements for DNA synthesis in the salt-treated nuclear systems were found to be similar to those in the isolated nuclear system (Tanuma et al., 1980).

Products synthesized in isolated nuclei and 0.2 M KCl and 0.3 M KCl treated nuclei were analyzed by sedimentation in alkaline sucrose gradients. As shown in Figure 2A, in isolated nuclei, the product distributed in both short fragments and high molecular weight DNA fractions. The product in the high molecular weight DNA fractions increased with pulse-labeling time. Whereas in 0.2 M KCl treated nuclei (Figure 2B), the product was found predominantly in the short fragments. Little capacity to synthesize short fragments was observed in 0.3 M KCl treated nuclei (Figure 2C).

Reconstitution of Nuclear Systems. Reconstitution experiments were performed by using salt-treated nuclei (SN-x) and nuclear extracts (NE-y) (Table I). DNA synthesizing activity in 0.2 M KCl treated nuclei (SN-0.2) was enhanced by the addition of their complementary nuclear extract (NE-0.2; extracted from isolated nuclei with 0.2 M KCl), whereas in the case of NE-0.3~0.2 (extracted from 0.2 M KCl treated nuclei with 0.3 M KCl), little enhancement of the activity was observed. DNA synthesis in the reconstituted nuclear system (SN-0.2/NE-0.2) was increased with the amount of NE-0.2 up to 3 nuclear equiv. When NE-0.3 (extracted from isolated nuclei with 0.3 M KCl) was added back to 0.3 M KCl treated nuclei (SN-0.3), DNA synthesizing activity was restored in the 0.3 M KCl treated nuclei. The activity attained by the addition of 3 nuclear equiv of the extract was about 80% of that in isolated nuclei. DNA synthesizing activity reconstituted in SN-0.3 by the addition of NE-0.3~0.2 was dependent upon the amount of the extract. The activity was reached about 50% of that in isolated nuclei at 2 nuclear equiv. NE-0.2 had a slight stimulatory effect on DNA synthesis in SN-0.3. All the nuclear extracts had almost no endogenous activity.

Characterization of Reconstituted Nuclear Systems. For characterization of the two reconstituted nuclear systems,

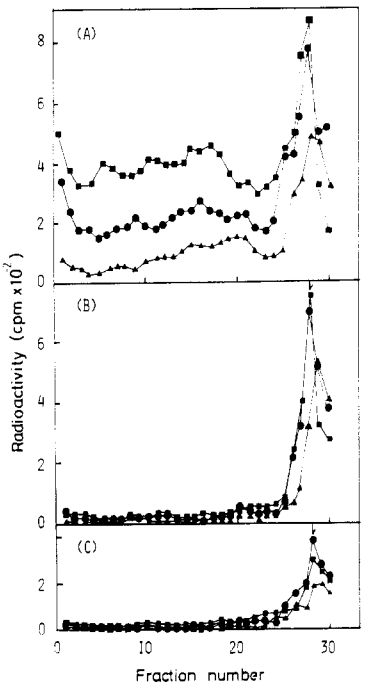

FIGURE 2: Alkaline sucrose gradient analysis of the products synthesized in isolated, 0.2 M KCl treated and 0.3 M KCl treated nuclei. Isolated nuclei (A), 0.2 M KCl treated nuclei (B), and 0.3 M KCl treated nuclei (C) were pulse labeled with [^{3}H]dTTP for 3 (▲), 5 (●), and 10 min (■) at 37 °C as descibed under Materials and Methods. The nuclei were lysed by the addition of alkaline lysis solution and then centrifuged in alkaline sucrose gradients as described under Materials and Methods. The arrow indicates the location of ^{14}C-labeled DNA of nucleosome monomer from HeLa S3 cells (4–5 S). The top of the gradient is at the right. The direction of sedimentation is to the left.

Table I: Effect of Nuclear Extracts on DNA Synthesis in Isolated and Salt-Treated Nuclei[a]

nuclear extract	amount of nuclear extract (×10^6 nuclear equiv)	DNA synthesizing activity (%) intact nuclei	SN-0.2[b]	SN-0.3[c]
none		100[g]	53	5
NE-0.3[d]	1	111	63	28
	2	–[h]	95	69
	3	121	101	81
	4	–	93	71
NE-0.2[e]	1	98	65	4
	2	–	78	13
	3	115	85	18
	4	–	88	15
NE-0.3~0.2[f]	1	109	57	21
	2	–	57	46
	3	118	59	53
	4	–	58	49

[a] Nuclei, salt-treated nuclei, and nuclear extracts were prepared as described under Materials and Methods. 1 × 10^6 nuclei or salt-treated nuclei were incubated at 37 °C for 10 min in the standard reaction mixture as described under Materials and Methods except that the concentration of KCl in the reaction mixture was changed so as to make a final concentration of 100 mM after the addition of nuclear extract. [b] SN-0.2, 0.2 M KCl treated nuclei. [c] SN-0.3, 0.3 M KCl treated nuclei. [d] NE-0.3, 0.3 M KCl nuclear extract. [e] NE-0.2, 0.2 M KCl nuclear extract. [f] NE-0.3~0.2, 0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei. [g] 100% corresponds to 5.2 pmol of dTMP incorporation. [h] Minus sign (–), not done.

SN-0.2/NE-0.2 and SN-0.3/NE-0.3~0.2, optimal conditions and requirements for DNA synthesis and the effects of inhibitors were compared with those of isolated and 0.2 M KCl treated nuclei (Table II). These properties in SN-0.2/NE-0.2 and SN-0.3/NE-0.3~0.2 were found to be identical with those in isolated nuclei and 0.2 M KCl treated nuclei, respectively. Optimal pH for DNA synthesis in all four systems was around 7.8. All four deoxyribonucleoside triphosphates were required for the maximal activities. Removal of one or more of the deoxyribonucleoside triphosphates resulted in marked reduction of the activities. Optimal concentration of ATP was around 4 mM. By the omission of ATP, the activities were less than 10% of the maxima. At an ATP concentration of 4 mM, the optimal magnesium concentration was 5 mM. In the absence of magnesium ion, the activities were less than 5% of the maxima. Addition of 1 mM araCTP or 10 mM *N*-ethylmaleimide almost completely inhibited the activities. Aphidicolin, a specific inhibitor of DNA polymerase α (Ohashi et al., 1978), also inhibited the activities, whereas ddTTP had essentially no effect on the activities at ddTTP/dTTP concentration ratio of 1.5 and 3. Ribonucleoside triphosphates (CTP, GTP, and UTP) at a final concentration of 0.1 mM each slightly stimulated the activities in the presence of optimal concentration of ATP and four deoxyribonucleoside triphosphates.

Table II: Requirements for and Effects of Inhibitors on DNA Synthesis in Reconstituted Nuclear Systems[a]

condition	DNA synthesizing activity (%) intact nuclei	SN-0.2[b]	SN-0.2/NE-0.2[b]	SN-0.3/NE-0.3~0.2[b]
complete[c]	100	100	100	100
deletion				
dATP	12	23	13	23
dGTP	12	22	13	24
dCTP	9	22	11	22
dATP, dGTP, dCTP	2	4	13	4
ATP	3	8	4	8
Mg	2	5	2	4
addition				
ara CTP				
0.3 mM	4	7	5	8
1.0 mM	3	4	4	4
ddTTP				
30 μM	98	87	93	91
60 μM	91	79	91	85
aphidicolin				
1 μg/mL	7	4	8	3
3 μg/mL	2	1	4	1
CTP, GTP, UTP (0.1 mM each)	109	121	111	118

[a] DNA synthesizing activity of 1 × 10^6 nuclei was measured as described in Table I. 3 × 10^6 nuclear equiv of nuclear extracts was added to the reconstituted systems and the concentration of KCl was adjusted as described in Table I. [b] Abbreviations are the same as in Table I. [c] Values are normalized with respect to the activity of each complete system as 100%.

Products synthesized in the two reconstituted nuclear systems were analyzed by sedimentation in alkaline sucrose gradients. The product synthesized in the reconstituted nuclear system consisting of SN-0.2 and NE-0.2 was found in both short fragments and high molecular weight DNA fractions (Figure 3A). In the reconstituted nuclear system, SN-0.3/NE-0.3~0.2, only short fragments were synthesized (Figure 3B). For examination of whether the short fragments synthesized in SN-0.2 and SN-0.3/NE-0.3~0.2 correspond to Okazaki fragments that should be joined to high molecular weight DNA, pulse–chase experiments were performed (Table III). When 0.2 M KCl treated nuclei were pulse labeled with

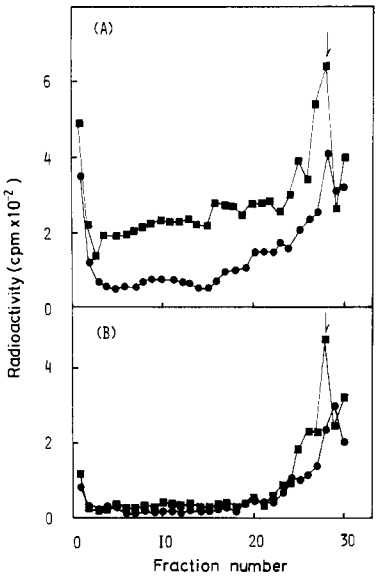

FIGURE 3: Alkaline sucrose gradient analysis of the products synthesized in reconstituted nuclear systems. Reconstituted nuclear systems consisting of 0.2 M KCl treated nuclei (1×10^6) and 0.2 M KCl nuclear extract (3×10^6 nuclear equiv) (A) or 0.3 M KCl treated nuclei (1×10^6) and 0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei (3×10^6 nuclear equiv) (B) were pulse labeled for 3 (●) and 10 (■) min at 37 °C. Preparation of samples and alkaline sucrose gradient centrifugation were performed as described under Materials and Methods. The same marker as in Figure 2 was used. The top of the gradient is at the right. The direction of sedimentation is to the left.

Table III: Ligation of Short Fragments Synthesized in 0.2 M KCl Treated Nuclei and a Reconstituted Nuclear System in the Presence of 0.2 M KCl Nuclear Extract[a]

		SN-0.2[b]		SN-0.3/NE-0.3~0.2[b]	
	time (min)	short[c] fragment [cpm (%)][d]	high molecular[c] weight DNA [cpm (%)]	short fragment [cpm (%)]	high molecular weight DNA [cpm (%)]
pulse	3	1502 (90)	159 (10)	982 (91)	108 (9)
chase	10	1288 (71)	540 (29)	951 (82)	307 (18)
	30	831 (42)	1138 (58)	1058 (67)	531 (33)
	30[e]	1102 (63)	625 (34)	932 (83)	189 (17)

[a] Nuclei or reconstituted nuclei (1×10^6) were pulse labeled with [^{3}H]dTPP for 3 min at 37 °C as described under Materials and Methods and chased with a 130-fold excess unlabeled dTTP in the presence of 3×10^6 nuclear equiv of 0.2 M KCl nuclear extract. Centrifugation was performed as described under Materials and Methods. [b] Abbreviations are the same in Table I. [c] The radioactivity of short fragments and high molecular weight DNA fractions were determined by summing the count in fraction numbers 1-6 and 7-30 from the top of the gradient, respectively. [d] %, % of the total count in the gradient. [e] Chased in the absence of 0.2 M KCl nuclear extract.

[^{3}H]dTTP for 3 min and then followed by chasing for 10 min with a 130-fold unlabeled dTTP in the presence of NE-0.2, about 20% of short fragments were converted to high molecular weight DNA. Increased synthesis of high molecular weight DNA was observed after a 30-min chase. The product synthesized in the reconstituted nuclear system, SN-0.3/NE-0.3~0.2, was also converted to high molecular weight DNA by chasing in the presence of NE-0.2. No decrease in the total radioactivity was observed in the both systems during the chase periods.

Analysis of ATP Requirement in the Process of Okazaki Fragment Synthesis. ATP requirement in the process of Okazaki fragment synthesis was analyzed with SN-0.2 which

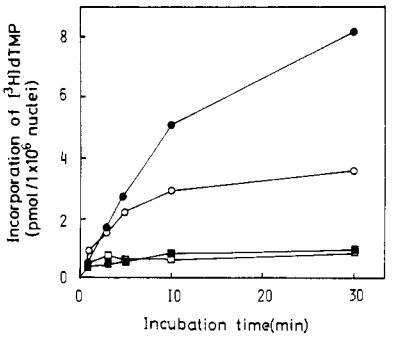

FIGURE 4: Time course of DNA synthesis in isolated nuclei and 0.2 M KCl treated nuclei in the presence or absence of ATP. Isolated (●, ■) and 0.2 M KCl treated (○, □) nuclei were incubated in the standard reaction mixture as described under Materials and Methods with (●, ○) or without (■, □) ATP.

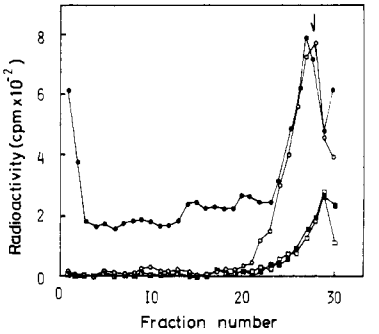

FIGURE 5: Alkaline sucrose gradient analysis of newly synthesized DNA in isolated nuclei and 0.2 M KCl treated nuclei in the presence or absence of ATP. Isolated (●, ■) and 0.2 M KCl treated (○, □) nuclei were pulse labeled with [^{3}H]dTTP in the presence (●, ○) or absence (■, □) of ATP for 10 min at 37 °C as described under Materials and Methods. The nuclei were lysed by the addition of alkaline lysis solution and centrifuged in alkaline sucrose gradients as described under Materials and Methods. The same marker as in Figure 2 was used. The top of the gradient is at the right, and the direction of sedimentation is to the left.

synthesized Okazaki fragments only. SN-0.2 showed ATP-dependent DNA synthesis. The dependency curve was similar to that of isolated nuclei. ATP (4–8 mM) gave maximal activity. In the absence of ATP, the activity was less than 10% of the maximum. Figure 4 shows typical kinetics of DNA synthesis in SN-0.2 and isolated nuclei in the presence or absence of ATP. The initial rate of DNA synthesis in SN-0.2 in the presence of ATP was the same as that in isolated nuclei, but the synthesis was ceased within 10 min, while in the absence of ATP, DNA synthesis ceased after a few minutes and was followed by a plateau in both nuclei.

Products synthesized in isolated nuclei and SN-0.2 in the presence or absence of ATP were analyzed by sedimentation in alkaline sucrose gradients. As shown in Figure 5, in isolated nuclei, newly synthesized DNA distributed in short fragments and high molecular weight DNA fractions in the presence of ATP, whereas in the absence of ATP, products were not found in high molecular weight DNA fractions. Furthermore, the amount of short fragments synthesized in the absence of ATP was much lower than that in the presence. In SN-0.2, the products synthesized in the presence of ATP sedimented in short fragments, and little radioactivity was found in high molecular weight DNA fractions. The amount of short fragments synthesized in the absence of ATP was lower than in the presence. The ATP required for the synthesis of Okazaki fragments in SN-0.2 was substituted by dATP and ADP completely and partially, respectively. Other ribonucleotides tested were not so effective. Products synthesized in SN-0.2 in the presence of 4 mM ATP, dATP, or ADP were

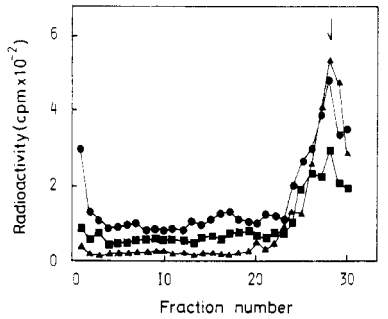

FIGURE 6: Alkaline sucrose gradient analysis of newly synthesized DNA in a reconstituted nuclear system consisting of 0.2 M KCl treated nuclei and the complementary nuclear extract in the presence of a low level of ATP and a high level of dATP or ADP. The reconstituted nuclei were incubated for 10 min at 37 °C as described in Figure 3 in the presence of 4 mM ATP (●), dATP (▲), or ADP (■) plus 200 μM ATP. The nuclei were prepared for alkaline sucrose gradient sedimentation as described under Materials and Methods. The same marker as in Figure 2 was used. The top of the gradient is at the right, and the direction of sedimentation is to the left.

compared by sedimentation in alkaline sucrose gradients. No significant difference was observed among the three nucleotides.

Role of ATP in the Joining Step of Okazaki Fragments. The role of ATP in the joining step of Okazaki fragments was analyzed with a reconstituted nuclear system, SN-0.2/NE-0.2. ADP (4 mM) or dATP (4 mM) was used to substitute for 4 mM ATP required for the synthesis of Okazaki fragments. Since the activity of DNA ligase optimized with lower concentrations of ATP (Söderhäll & Lindahl, 1976) than those required for DNA synthesis in SN-0.2, the concentration of ATP was set at 200 μM at which almost no stimulation of the DNA synthesis was observed. As shown in Figure 6, products synthesized in the reconstituted nuclei after a 10-min pulse in the presence of 4 mM ADP and 200 μM ATP distributed in short fragments and high molecular weight DNA fractions as in the presence of high level of ATP. In contrast, the ability to synthesize high molecular weight DNA was not restored in the reconstitituted nuclei by the combination of 4 mM dATP and 200 μM ATP.

Pulse–chase experiments were performed (Table IV) to confirm further that short fragments synthesized in the presence of high level of ADP can be joined in the presence of low level of ATP. SN-0.2 was pulse labeled with [^{3}H]dTTP in the presence of 4 mM ADP and followed by chasing with 130-fold unlabeled dTTP in the presence of NE-0.2 and 200 μM ATP. Considerable amount of short fragments synthesized with ADP were converted to high molecular weight DNA during a 10-min chase. No decrease in the total radioactivity was observed during the chase period.

Table IV: Ligation of Okazaki Fragments with a Low Level of ATP[a]

	incubation time (min)	Okazaki fragment[b] [cpm (% of total)]	high molecular weight[b] DNA [cpm (% of total)]
pulse	3	1621 (91)	153 (9)
chase	3	1446 (80)	355 (20)
	10	1198 (67)	581 (33)
	10[c]	1768 (89)	214 (11)

[a] 0.2 M KCl treated nuclei (1×10^6) were pulse labeled with [^{3}H]dTTP for 3 min in the reaction mixture as described under Materials and Methods except that 4 mM ADP was added in place of 4 mM ATP and chased with a 130-fold excess unlabeled dTTP in the presence of 3×10^6 nuclear equiv of 0.2 M KCl nuclear extract and 200 μM ATP. Centrifugation was performed as described under Materials and Methods. [b] The radioactivity of Okazaki fragments and high molecular weight DNA fractions were determined by summing the count in fraction numbers 1–6 and 7–30 from the top of the gradient, respectively. [c] 0.2 M KCl treated nuclei were pulse labeled and chased as described in footnote *a* except that they were chased in the absence of 200 μM ATP.

Discussion

In this report we have developed reconstituted nuclear systems using salt-treated nuclei which are defective in some steps of DNA replication. The ability of these systems to reconstitute processes of DNA replication by adding back nuclear salt extracts seems promising for the functional analysis of enzymes and factors participating in DNA replication.

Analysis of newly synthesized DNA revealed that isolated nuclei synthesized short fragments and high molecular weight DNA, whereas 0.2 M KCl treated nuclei (SN-0.2) synthesized short fragments only. The short fragments synthesized in SN-0.2 were converted to high molecular weight DNA in the presence of their complementary nuclear extract, NE-0.2 (Table III). Furthermore, the optimal conditions and requirements for the DNA synthesis in the reconstituted nuclear system were essential the same as those in intact nuclei (Table II). These results indicate that SN-0.2 retain the factors and structure needed for the synthesis of short fragments (Okazki fragments) but are deficient in some factors involved in the joining of these fragments. SN-0.2 and NE-0.2, therefore, may become a valuable "natural template" and a starting material, respectively, for the identification and purification of factors necessary to join Okazaki fragments.

The possibility that the short fragments synthesized in the reconstituted nuclear system, SN-0.3/NE-0.3~0.2, are Okazaki fragments which should be ligated to yield high molecular weight DNA was supported by the following observations. The properties of DNA synthesis in the reconstituted nuclear system closely resembled those in SN-0.2, and the short fragments synthesized in the reconstituted nuclear system were converted to high molecular weight DNA by chasing in the presence of NE-0.2. It appears that NE-0.3~0.2 contains some essential factors involved in the synthesis of Okazaki fragments, and 0.3 M KCl treated nuclei are a useful template for the identification of these factors.

Our previous observations with isolated nuclear system that a high level of ATP is required for the synthesis of Okazaki fragments and the joining step of the fragments is satisfied with a low level of ATP (Enomoto et al., 1981) were further confirmed by using SN-0.2 and a reconstituted nuclear system, SN-0.2/NE-0.2. In SN-0.2, a high level of dATP completely replaced a high level of ATP required for the synthesis of Okazaki fragments, indicating that a factor needed for the synthesis of Okazaki fragments uses dATP with equal efficiency as ATP.

Although the molecular basis of the requirement of high level of ATP for the synthesis of Okazaki fragments is as yet unknown, one possibility can be speculated that ATP participates in conformational changes of DNA into a form to permit the synthesis. It has been reported that DNA-dependent ATPases play important roles in DNA replication of bacteria and phages. It is interesting that some of these enzymes from *Escherichia coli* can utilize dATP as well as ATP to unwind DNA (Geider & Hoffman-Berling, 1981). Recently, DNA-dependent ATPases have been isolated from eukaryotic cells (Hachmann & Lezius, 1976; Hotta & Stern, 1978; Assairi & Johnston, 1979; Boyer & Korn, 1980; Plevani et al., 1980; Watanabe et al., 1981; Cobianchi et al., 1981), and these

enzymes (Hachmann & Lezius, 1976; Boyer & Korn, 1980; Plevani et al., 1980; Watanabe et al., 1981) have been shown to hydrolyze dATP with equal efficiency as ATP. We have observed that there exists DNA-dependent ATPase activity in 0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei (unpublished result). This DNA-dependent ATPase is likely to be a candidate which requires a high level of ATP in the process of the synthesis of Okazaki fragments.

The resumption of the ability to synthesize high molecular weight DNA in SN-0.2/NE-0.2 by the combination of a high level of ADP and a low level of ATP (Figure 6, Table IV) may be due to the supplement of the cofactor of DNA ligase because ADP cannot substitute for ATP as a cofactor of DNA ligase (Zimmerman & Levin, 1975). The inability to synthesize high molecular weight DNA in the presence of a high level of dATP may be due to the inhibition of the activity of DNA ligase. This interpretation is consistent with the observation of Katouzian & David (1978) that dATP is a potent inhibitor of DNA ligase. From these results, it was clearly indicated that the joining step of Okazaki fragments can be satisfied with a low level of ATP, which plays a role as a cofactor of DNA ligase.

DNA syntheses in the reconstituted nuclear systems were inhibited by aphidicolin, a specific inhibitor of DNA polymerase α, but not by ddTTP, a potent inhibitor of DNA polymerase β and γ. These observations indicate that DNA syntheses in the reconstituted systems are catalyzed by DNA polymerase α. As described in the following paper (Enomoto et al., 1983), it was observed that NE-0.3~0.2 contained one form of DNA polymerase α (form I) and that NE-0.2 was enriched with another form of DNA polymerase α (form II). Possible roles of these DNA polymerases in the processes of DNA replication will be discussed in the following paper.

Registry No. ATP, 56-65-5; dATP, 1927-31-7; ADP, 58-64-0; araCTP, 13191-15-6; ddTTP, 611-60-9; aphidicolin, 38966-21-1.

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