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Poly (ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase α, δ and ε in vitro

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Abstract The influence of poly (ADP-ribose) polymerase (PARP) and poly ADP-ribosylation on DNA synthesis supported by human replicative DNA polymerase (DNA pol) α, δ, and ε has been examined using the replication system containing poly(dA)$_{4500}$–oligo(dT)$_{12-18}$ as the template primer. PARP alone inhibited the pol activities in a dose-dependent manner even in the presence of the accessory factors for DNA pol δ, proliferating cell nuclear antigen (PCNA) and activator 1 (A1; RF-C). Both DNA pol α and ε activities were decreased approximately 10-fold under the poly ADP-ribosylating condition. In contrast, DNA synthesis by DNA pol δ holoenzyme was not affected by poly ADP-ribosylation like prokaryotic DNA pol's. The analysis of poly(dT) formed by DNA pol α and ε indicated that poly ADP-ribosylation mainly reduced the frequency of replication. These observations suggest a possibility that PARP acts as a negative regulator for the initiation of DNA replication upon cellular DNA damage.

Key words: Poly ADP-ribose polymerase; Poly ADP-ribosylation; Eukaryotic DNA replication; Human DNA polymerase α, δ and ε

1. Introduction

In the last decade, many details of mammalian DNA replication have been elucidated using the cell-free system for simian virus 40 (SV40) DNA replication containing SV40 DNA, purified SV40 T antigen, and HeLa cell extract (for reviews see [1–3]). Biochemical studies of the factors which are essential for this system have allowed us to identify and purify several cellular replication proteins, including DNA polymerase (DNA pol) α-primase complex [4], DNA pol δ [5], proliferating cell nuclear antigen (PCNA) [6], activator 1 (A1 [7], also identified as RF-C [8]), human single-stranded DNA binding protein (HSSB, also known as replication protein A, RP-A) [9,10], and topoisomerase I and II [11]. The roles of two DNA pol's (α and δ) in the initiation and elongation of SV40 DNA replication have been elucidated using two distinctive systems, i.e. the mono- and di-polymerase systems, reconstituted with the purified proteins [11,12]. During the initiation of replication, DNA pol α-primase complex interacts with T antigen and HSSB at the origin of replication and initiates the synthesis of RNA primers and subsequent Okazaki fragments from lagging strand synthesis in the presence of ribo- and deoxyribonucleoside triphosphates [13,14]. Highly efficient and processive synthesis of leading strand can be supported by DNA pol δ holoenzyme (DNA pol δ, PCNA, and A1) [5,15,16]. In addition to DNA pol α and δ, DNA pol ε was also purified as the third DNA pol from HeLa cell extract [17,18]. So far, it has reported that DNA pol ε (pol II) is essential for DNA replication in *S. cerevisiae* [19].

Previously, I identified poly (ADP-ribose) polymerase (PARP) to a cellular factor inhibiting SV40 DNA replication in vitro, and clarified its actions in detail [20]. PARP is an abundant nuclear protein and polymerizes poly (ADP-ribose) chains using NAD by binding to the DNA ends [21,22]. Although the physiological role of poly ADP-ribosylation is unknown, PARP has been implicated in DNA-dependent reactions such as DNA repair [23] and DNA replication [24]. In this report, I have examined the influence of PARP and poly ADP-ribosylation on the DNA synthesis supported by human replicative DNA pol α, δ, and ε.

2. Materials and methods

2.1. Materials

Radioactive materials were purchased from Amersham. Dithiothreitol (DTT), dNTPs, rNTPs, and creatine phosphokinase were from Boehringer Mannheim; NuSieve 3:1 agarose was from FMC Bioproducts; T4 DNA pol was from Toyobo (Tokyo); the Klenow fragment of *E. coli* DNA pol I was from Takara Shuzo (Kyoto); other chemicals were from Sigma and Wako Chemical Inc. (Tokyo). Poly(dA)$_{4500}$ and oligo(dT)$_{12-18}$ were obtained from Life Science Inc. (Florida) and Pharmacia LKB, respectively. A 1-kb DNA ladder was from Gibco BRL.

2.2. Preparation of proteins and enzymes

The HeLa whole cell extract with 0.2 M NaCl was fractionated into three parts (0.2 M NaCl pass-through, 0.4 M NaCl eluate, 0.7 M NaCl eluate fractions) by phosphocellulose column chromatography, for the further purification of cellular replication proteins described below.

PARP (EC 2.4.2.30) was purified to homogeneneity from the phosphocellulose 0.7 M NaCl fraction through the following eight steps (phosphocellulose, Q Sepharose, single-stranded DNA cellulose, NAD-agarose, blue-Sepharose, hydroxylapatite, Superose 6 HR10/30 FPLC columns, and glycerol density gradient centrifugation) as described previously [20]. PCNA (7100 units/mg protein) was assayed and purified from the phosphocellulose pass-through fraction as described previously [25].

DNA pol α, δ, and ε were purified from the phosphocellulose 0.4 M NaCl fraction. The activity of each DNA pol α, δ, and ε was assayed as described previously [5,11,18]. The phosphocellulose 0.4 M NaCl fraction was loaded onto a DEAE-Sepharose CL-6B column equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM DTT, and the protease inhibitors 0.1 mM

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Abbreviations: PARP, poly (ADP-ribose) polymerase; HSSB, human single-stranded DNA binding protein (identical to replication protein A, RP-A); SV40, simian virus 40; T antigen, virus-encoded large tumor antigen; PCNA, proliferating cell nuclear antigen; A1, activator 1 protein (identical to replication factor C, RF-C); DNA pol, DNA polymerase; BSA, bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol; kDa, kilodalton; kb, kilo base; nt, nucleotide.

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PMSF, 0.2 μg/ml antipain, 0.1 mg/ml leupeptin, 0.1 mM benzamidine) containing 50 mM NaCl. The proteins were eluted with a 2-l gradient of 0.05–0.4 M NaCl in buffer A. The peak of DNA pol δ activity was eluted at 130 mM NaCl, and that of DNA pol α was co-eluted with DNA pol ε at 180 mM NaCl. DNA pol δ fraction was directly loaded onto a phosphocellulose column equilibrated with buffer A containing 0.2 M NaCl, and eluted with a 0.6-l gradient of 0.2–0.5 M NaCl in buffer A. Half of the active fractions eluted at 0.25–0.35 M NaCl were directly loaded onto a hydroxylapatite column equilibrated with buffer B (10% glycerol, 0.05% Triton X-100, 1 mM DTT, protease inhibitors) containing 20 mM potassium phosphate, pH 7.5. The activity of DNA pol δ was eluted at 0.18–0.23 M potassium phosphate. The active fractions were diluted 2-fold with buffer B containing 20 mM potassium phosphate, and were loaded onto a heparin-Sepharose column equilibrated with buffer C (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 15% glycerol, 0.05% Triton X-100, protease inhibitors) containing 0.1 M KCl, and the activity of DNA pol δ was eluted with a 24 ml gradient of 0.1–0.6 M KCl. The active fractions (peak fraction, 1.9 mg protein) were pooled and dialyzed against a dialysis buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10% glycerol, 20% sucrose, 0.25 M KCl, 0.05% Triton X-100, protease inhibitors). DNA pol δ (2467 units/mg protein in the peak fraction) was held as a stock solution with 0.1 μl/ml bovine serum albumin (BSA) at −80°C. The DNA pol δ activity was totally dependent upon the presence of PCNA, and was stimulated by A1.

DNA pol α-primase complex (3200 units/mg protein of DNA pol α) was purified from the DEAE-Sepharose active fractions of DNA pol α and ε by SJK237 monoclonal antibody column chromatography as described previously [11]. DNA pol ε was purified from the DNA pol α-depleted fractions from a SJK237 affinity column as follows. The fractions were loaded onto a double column containing a SJK287 monoclonal antibody column on top of a phosphocellulose column equilibrated with buffer A containing 0.2 M NaCl. After detaching an affinity column, the proteins were eluted with a 440-ml gradient of 0.2–0.5 M NaCl. The active fractions at 0.27 M NaCl were directly loaded onto a hydroxylapatite column equilibrated with buffer B containing 25 mM potassium phosphate, pH 7.5. The activity of DNA pol ε was eluted at 0.08–0.18 M with a 160-ml gradient of 0.025–0.35 M potassium phosphate in buffer B. The active fractions were diluted with an equal volume of buffer B containing 25 mM potassium phosphate, and loaded onto a heparin-Sepharose column equilibrated with buffer C containing 0.1 M KCl. DNA pol ε activity was eluted at 0.35 M with a linear gradient of 0.1–0.5 M KCl in buffer C. The active fractions were diluted 3-fold with buffer C, and then loaded onto a Q-Sepharose column equilibrated with buffer C containing 0.1 M KCl. The activity of DNA pol ε was eluted at 0.4 M KCl with a 26-ml gradient of 0.1–0.5 M KCl in buffer C. The active fractions were concentrated by step-wise hydroxylapatite column chromatography followed by a dialysis against buffer D (20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.05% Triton X-100, protease inhibitors) containing 20% sucrose and 0.2 M NaCl. An aliquot of the concentrated fraction (0.5 ml; 625 μg protein) was loaded onto a Sephacryl S-400 gel-filtration column (18 ml bed, 0.7 × 50 cm diam.) equilibrated with buffer D containing 0.2 M NaCl. The active fractions were pooled and dialyzed against a dialysis buffer (20 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10% glycerol, 20% sucrose, 0.1 M KCl, 0.05% Triton X-100, protease inhibitors). DNA pol ε (14 μg protein in the peak fraction; 15,930 units/mg protein) was held as a stock solution with 0.1 mg/ml BSA at −80°C.

Activator 1 (1100 units/mg protein) was purified from the phosphocellulose 0.7 M NaCl fraction according to procedure 2 (phosphocellulose, hydroxylapatite, single-stranded DNA cellulose, Q-Sepharose, glycerol gradient centrifugation) of Lee et al. [7]. A1 activity was assayed by its ability to stimulate DNA pol δ activity in the presence of SSB, PCNA, and ATP, as described previously [7].

2.3. Protein assay

Protein concentrations were measured using the Bradford dye reagent (Bio-Rad) with BSA as the standard.

2.4. Assay for DNA polymerases in the poly(dA)–oligo(dT) system

The activity of each DNA pol was measured under the same conditions as for the SV40 in vitro replication assay using poly(dA)$_{4500}$–oligo(dT)$_{12-18}$ (20:1 ratio as nucleotides) as a template. Reaction mixtures (30 μl) contained 40 mM creatine phosphate (di-Tris salt, pH 7.7), 1 μg of creatine phosphokinase, 7 mM $MgCl_2$, 1 mM DTT, 5 μg of BSA, 0.2 μg of poly(dA)$_{4500}$–oligo(dT)$_{12-18}$, 4 mM ATP, and 66.6 μM [^{3}H]dTTP (412 cpm/pmol). DNA pol α-primase complex was used as DNA pol α throughout the experiments. DNA pol δ activity was assayed in the presence of PCNA (0.1 μg). Purified A1 (Q-Sepharose peak fraction; 0.1 unit) was added for assaying the activity as DNA pol δ holoenzyme. The Klenow fragment of DNA pol I and T4 DNA pol were obtained from the companies mentioned above. Approximately 0.1 unit of enzymes were used for the experiments. For examining the effects of PARP or poly ADP-ribosylation on each DNA pol activity, a reaction mixture without dTTP was preincubated in the presence of various amounts of PARP with or without 2 mM NAD prior to the reaction at 25°C for 20 min. Then, the reaction mixture supplemented with [^{3}H]dTTP was incubated at 37°C for 30 min. The amount of acid-insoluble dTMP was determined for measuring the remaining DNA pol activity.

2.5. Product analysis

Reaction mixtures (30 μl) containing 0.2 μg of poly(dA)$_{4500}$–oligo(dT)$_{12-18}$ (40:1) and 66.6 μM [α-^{32}P]dTTP (3700 cpm/pmol), were used. The reaction mixtures were preincubated with PARP and NAD at 25°C for 20 min. Then, the reactions were carried out in the presence of [α-^{32}P]dTTP at 37°C for 20 min. The amount of synthesis was determined by measuring the acid-insoluble materials formed using a small aliquot. The remaining mixtures were supplemented with 20 mM EDTA, 1% SDS, 40 μg of yeast tRNA, and digested with proteinase K (0.1 mg/ml) at 37°C for 30 min. DNA was purified by phenol/chloroform extraction and ethanol precipitation in the presence of 5 M ammonium acetate and 20 μg of glycogen. The products were analyzed by 3% alkaline agarose gel-electrophoresis in 30 mM NaOH and 1 mM EDTA at 3 V/cm for 12 h. The gel was dried on Whatman DE81 paper and then exposed to X-ray film (Kodak X-OMAT AR) at −80°C. A 1-kb DNA ladder, labeled at the 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP, was used for size markers. Throughout the experiments, comparable amounts of DNA pol α, DNA pol δ holoenzyme (DNA pol δ/PCNA/A1), and DNA pol ε, that supported the incorporation of 80–200 pmol dTMP into acid-insoluble materials in the system described above, were used.

2.6. Assay of poly ADP-ribosylation in the reactions

The poly ADP-ribosylation in the replication reactions containing PARP and NAD, was assayed by determining the incorporation of [^{3}H]NAD into acid-insoluble materials using parallel reactions with unlabeled dTTP [20].

3. Results

3.1. Influence of PARP and poly ADP-ribosylation on DNA replication by DNA pol α, δ, and ε in the poly(dA)–oligo(dT) system

The influence of PARP on DNA replication supported by DNA pol α, δ, and ε purified from HeLa cell extract was examined by measuring the synthesis of poly(dT) using the poly(dA)–oligo(dT) system containing each DNA pol and

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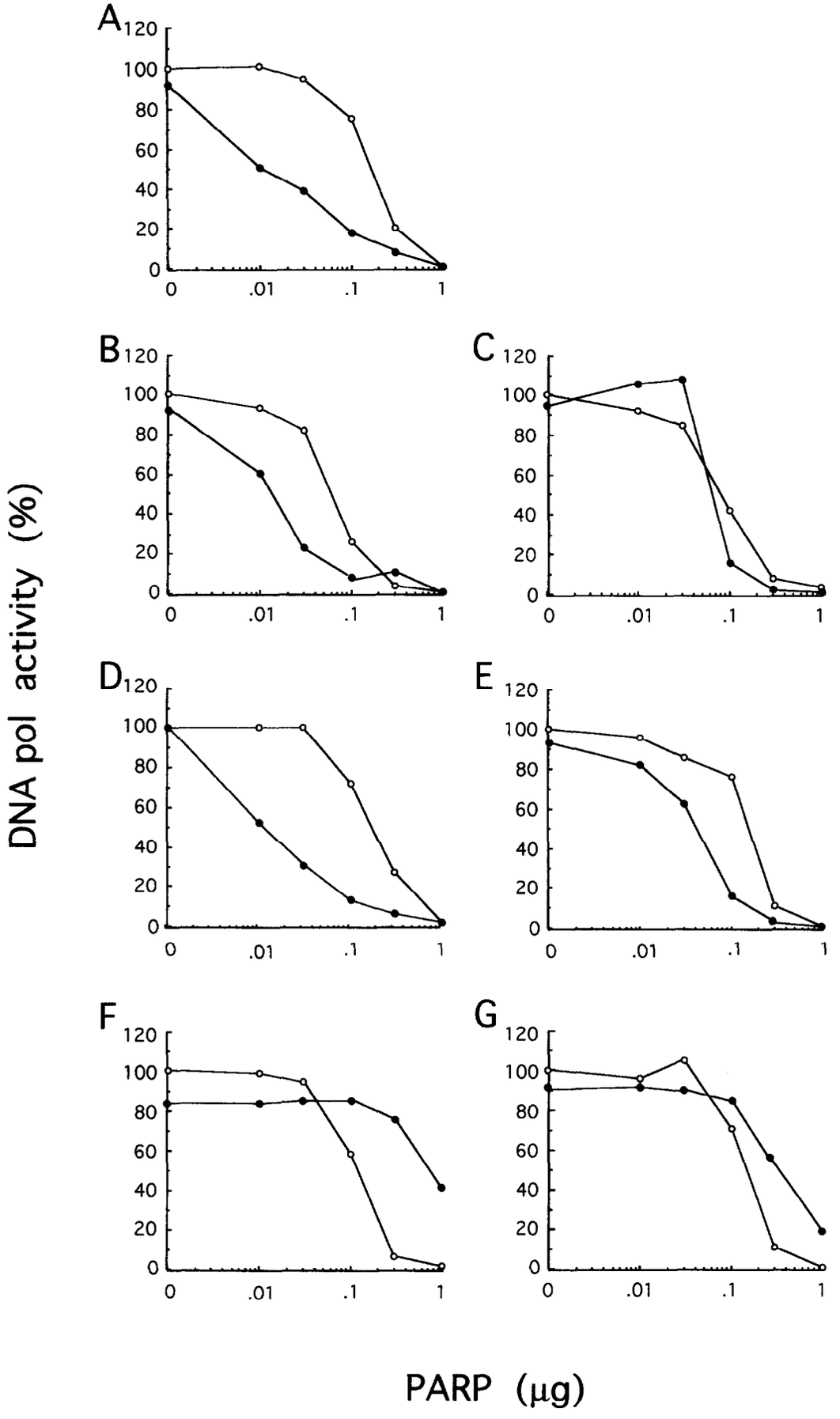
Fig. 1. The influence of PARP and NAD on poly(dT) synthesis supported by various DNA pol's. The reaction mixtures without dTTP contained approximately 0.1 unit of DNA pol α-primase complex (A), DNA pol δ with 0.1 μg of PCNA (B), DNA pol δ with PCNA and 0.1 unit of A1 (C), DNA pol ε (D), DNA pol ε with PCNA and A1 (E), the Klenow fragment of DNA pol I (F), and T4 phage DNA pol (G), respectively. After preincubation in the presence (●) or in the absence (○) of 2 mM NAD at 25°C for 20 min, the reactions were carried out with dTTP at 37°C for 30 min, and the DNA polymerase activity was determined by measuring the incorporation of dTMP as described in section 2. The 100% value in each assay was equal to 161 (A), 67 (B), 117 (C), 100 (D), 161 (E), 60 (F), 86 pmol (G) of the dTMP incorporation, respectively.


A
B
C
D
E
F
G
120
100
80
60
40
20
0
0
.01
.1
1
DNA pol activity (%)
PARP (μg)

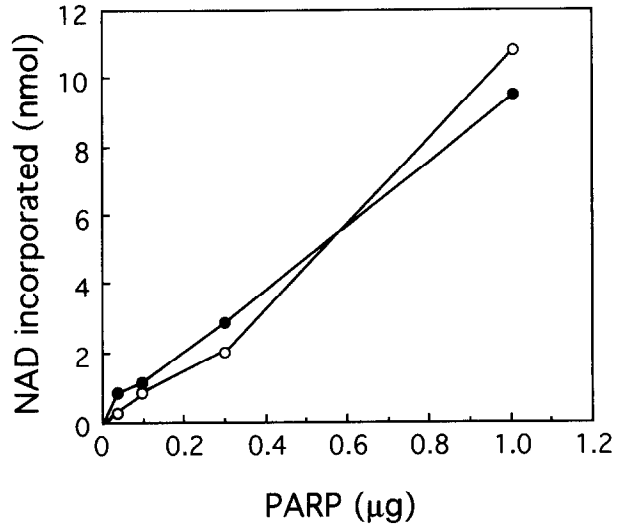


Fig. 2. The synthesis of poly (ADP-ribose) in the poly(dA)–oligo(dT) system. Parallel reactions containing unlabeled dTTP and 2 mM [^{3}H]NAD were performed in the presence (●) or the absence (○) of DNA pol α as described in the legend for Fig. 1. The incorporation of [^{3}H]NAD into acid-insoluble materials was determined as described in section 2.

various amounts of PARP. Parallel reactions containing 2 mM NAD were performed simultaneously to examine the effects of poly ADP-ribosylation on DNA replication. The results are summarized in Fig. 1. Poly(dT) syntheses by each human DNA pol were similarly inhibited by PARP alone according to the amount of added PARP. The amounts of PARP inhibiting 50% of the DNA pol activity were 170 ng (DNA pol α; Fig. 1A), 50 ng (DNA pol δ/PCNA; Fig. 1B), and 160 ng (DNA pol ε; Fig. 1D), respectively. Accessory factors (A1 and PCNA) did not affect the inhibition of DNA synthesis supported by DNA pol δ/PCNA (Fig. 1B and C) or DNA pol ε (Fig. 1D and E). In the presence of NAD, the activity of each DNA pol decreased 5- to 10-fold by PARP. Approximately 10 ng of PARP was sufficient to inhibit 50% of the pol activities (Fig. 1A, B, and D). In contrast, the addition of NAD did not affect the suppression of DNA pol δ/PCNA activity by PARP in the presence of A1 (compare B with C in Fig. 1). The activity of DNA pol ε with A1 and PCNA also resisted suppression 5-fold more than that of DNA pol ε alone under the same conditions (compare D with E in Fig. 1). Furthermore, the inhibition of DNA synthesis by prokaryotic DNA pol's, the *E. coli* Klenow fragment and T4 DNA pol by PARP alone was indistinguishable from those of eukaryotic DNA pol's. The addition of NAD in the reaction, however, restored DNA synthesis slightly (Fig. 1F and G).

Simultaneously, the amount of poly (ADP-ribose) formed in the reactions containing 2 mM NAD and PARP with or without DNA pol α, was determined by measuring the incorporation of [^{3}H]NAD (Fig. 2). Automodification of PARP accounted for over 90% of the total NAD incorporation. Similar results were obtained from the combination of PARP and other DNA pol's (data not shown).

3.2. Analysis of the poly(dT) synthesized in the poly(dA)–oligo(dT) system by alkaline agarose gel-electrophoresis

The products formed in the poly(dA)–oligo(dT) system were analyzed to clarify the mechanism of inhibition by PARP and poly ADP-ribosylation. Reaction mixtures containing each DNA pol and the indicated amounts (0, 20, and 100 ng) of PARP were incubated with or without 2 mM NAD, and the [^{32}P]poly(dT) formed was analyzed by alkaline agarose gel-electrophoresis. DNA pol α synthesized poly(dT) with 75–300 nt in length (Fig. 3, lane 1), and PARP reduced the initiation frequency of the poly(dT) synthesis as well as the size of products in the reactions (Fig. 3, lanes 2 and 3). The addition of NAD enhanced the suppression of both replication initiation and the length of products (Fig. 3, lanes 2 and 5). In the case of DNA pol δ and ε, PARP alone markedly reduced only the initiation of poly(dT) synthesis whether in the presence or in the absence of accessory factors (Fig. 3, lanes 7–9, lanes 13–15, lanes 19–21, lanes 25 and 26). The addition of NAD to the reaction mixture containing PARP (20 ng) and DNA pol ε suppressed the incorporation of dTMP from 37 pmol to 2.6 pmol. The mean size of products formed by DNA pol ε, however, only decreased from 400 nt to 250 nt (Fig. 3, lanes 20 and 23). Thus, the initiation of replication by DNA pol ε was dominantly suppressed under the poly ADP-ribosylating conditions. In contrast, the length of products formed in the reactions with DNA pol δ was not affected either in the presence or in the absence of NAD (Fig. 3, lanes 10–12, lanes 16–18).

4. Discussion

In this report, I examined the effects of PARP and poly ADP-ribosylation on DNA synthesis supported by eukayotic DNA pol α, δ, and ε using the simplified system containing the multi-primed template, poly(dA)–oligo(dT). It was observed that PARP alone suppressed DNA synthesis in a dose-dependent manner, and that the addition of NAD markedly enhanced the suppression of DNA synthesis (Fig. 1). The inhibition of replication by PARP alone is apparently due to the blocking of the 3′ hydroxyl ends of DNA chains by virtue of intrinsic DNA binding ability as previously reported [20]. As for the influence of PARP alone on DNA pol α activity, Simbulan et al. [26] recently reported discrepant result. They indicated that PARP stimulates DNA pol α activity by specific physical association in the system containing high concentrations of the activated DNA as template. Under their conditions, PARP can not block all of DNA ends and facilitates the associated DNA pol α to recognize DNA ends on the bound template rather than inhibits DNA pol α activity by blocking DNA ends. In contrast, an excess amount of PARP (in the case of 0.8 μg protein, approximately 6.5 pmol) was added into the assay system used here, which contains approximately 2.0 pmol of 3′ OH-ends of the poly(dA)–oligo(dT) template, to observe the inhibition of DNA pol α activity. The discrepancy may be accounted for by the difference in the concentration of the DNA ends used in the experiments, since they also observed inhibition at a low level of DNA template primer [26].

Extensive synthesis of poly (ADP-ribose) depending upon the amount of added PARP was observed in the reaction mixture containing NAD (Fig. 2). On the other hand, the enhanced suppression of replication with PARP and NAD is caused by the poly ADP-ribosylation of each DNA pol due to the following two reasons. First, this enhancement of suppression was not observed in the presence of 10-fold excess of 3-aminobenzamide, which is a specific inhibitor of poly ADP-ribosylation (data not shown). Secondly, the autopoly ADP-ribosylation of

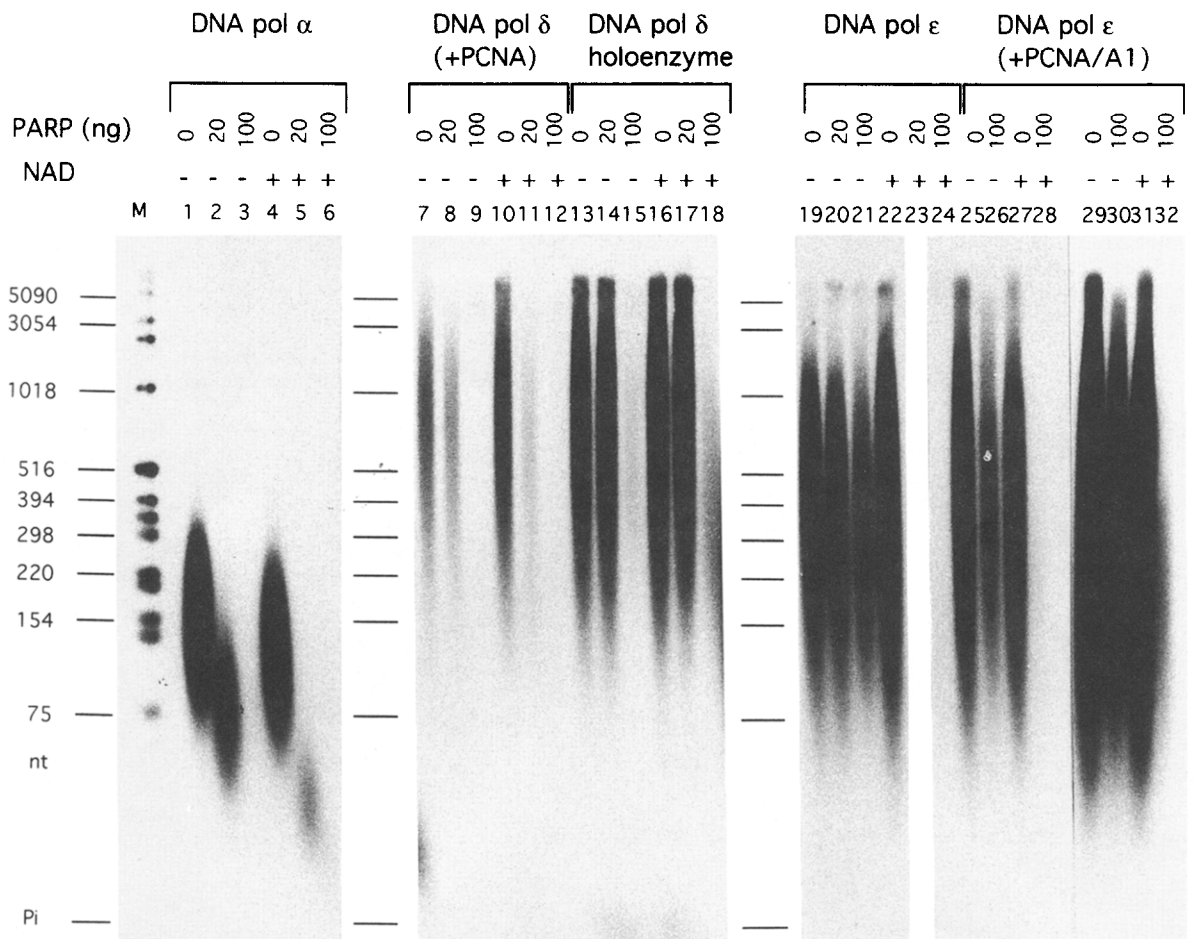


Fig. 3. The analysis of poly(dT) formed by human DNA pol's in the poly(dA)–oligo(dT) system by alkaline agarose gel-electrophoresis. The reaction mixtures contained 0.1 unit of DNA pol α-primase complex (lanes 1–6), DNA pol δ plus PCNA (0.1 μg) (lanes 7–12), DNA pol δ plus PCNA and A1 (0.08 unit) (lanes 13–18), DNA pol ε (lanes 19–24), and DNA pol ε plus PCNA and A1 (lanes 25–32), respectively. The reaction mixtures without dTTP were preincubated in the presence of various amounts of PARP and 2 mM NAD as indicated in the figure at 25°C for 20 min, and then were incubated at 37°C for 30 min with [^{32}P]dTTP. The [^{32}P]poly(dT) formed was fractionated by 3% NuSieve agarose gel-electrophoresis as described in section 2. A ^{32}P-labeled 1-kb DNA ladder was used for size markers (M). Lanes 29–32 are identical to lanes 25–28 except for the exposure time.

PARP reduces its DNA binding ability (data not shown, [27]). This should cause the restoration of DNA replication because PARP is released from the 3′ ends of DNA. DNA synthesis was, however, markedly suppressed under this condition. The inactivation of DNA pol α by poly ADP-ribosylation was also observed by other groups [28]. Interestingly, DNA synthesis by DNA pol δ holoenzyme was insensitive to the suppression by poly ADP-ribosylation (Fig. 1C). This observation can be accounted for by the difference in the ability of primer recognition and the processivity among DNA pol's in the presence of PCNA and A1. A1 can compete with PARP by virtue of its ability to recognize the 3′ ends of DNAs as previously reported [20]. Furthermore, only DNA pol δ/PCNA can efficiently utilize the primer ends bound to A1 for elongation [5]. DNA pol ε can also exhibit some processivity like DNA pol δ under high salt conditions [18], but it is unable to recognize the primers well. It is also suggested that the DNA pol's polymerizing DNA chains processively can escape from the inactivation by poly ADP-ribosylation because the size of products formed by DNA pol δ (or ε) is not reduced greatly (Fig. 3). Thus, it is possible that DNA pol δ holoenzyme can initiate DNA synthesis with PARP competing for DNA ends and complete the chain elongation by virtue of its high processivity. Furthermore, prokaryotic DNA pol's were found to be totally unaffected by poly ADP-ribosylation, possibly because of the absence of acceptor sites for the modification (Fig. 1F and G). Throughout the product analysis, it was clear that poly ADP-ribosylation mainly reduced the initiation frequency more than the chain elongation. This may suggest that the attached bulky poly ADP-ribose chains prevent the enzyme from accessing the template DNA.

Besides the inactivation of DNA pol α, δ, and ε, it was reported that poly ADP-ribosylation inhibits the activities of DNA pol α, pol β, terminal deoxytransferase, DNA ligase II [28], topoisomerase I [29–31], II [32], and Ca^{2+},Mg^{2+}-dependent endonuclease [33]. I have also reported the poly ADP-ribosylation of HSSB, which is essential for DNA replication and DNA repair in vivo and in vitro [20]. The functional significance of the poly ADP-ribosylation of these proteins involved in DNA metabolism is still unknown. Since DNA pol α is the sole DNA pol essential for the initiation of cellular DNA replication [34], the inactivation of DNA pol α by poly ADP-ribosylation could prevent cellular DNA synthesis. Furthermore, a number of observations have been suggested from the close relationship

between poly ADP-ribosylation and DNA repair [23]. Taken together, PARP could shut down the initiation of DNA replication via the inactivation of replicative DNA pol's by poly ADP-ribosylation when DNA is damaged. Thus, it is likely that PARP may play a physiologically important role as a negative regulator induced by DNA damage in mammalian cells.

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