The possible roles for polyamines in the initiation process of SV40 DNA replication *in vitro*

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Received May 14, 2007; Accepted July 25, 2007

Abstract. The polyamines are aliphatic cations which are present in millimolar concentrations in all mammalian cells, and are required for optimal growth of almost all cell types. In this study, the roles of polyamines in DNA replication *in vitro* and the mechanism by which polyamines affected DNA replication were examined using simian virus 40 DNA replication system *in vitro*. We found that polyamines inhibited DNA replication, but it is not clear at which stage this occurs. Spermidine inhibited the DNA cleavage by topoisomerase I at 8.0 mM, but stimulated its activity at 1.0 mM. Spermine also inhibited its activity at 4.0 mM, but stimulated at 1.0 mM. The ssDNA binding activity of replication protein A was slightly affected by polyamines. Polyamines, especially spermine, also significantly reduced polymerase α-primase activity at 133 μM. Taken together, we suggest that the major inhibition of SV40 DNA replication may be due to the inhibition of pol α-primase activity, and possible roles for polyamines in the initiation process are discussed.

Introduction

The polyamines spermidine and spermine and their precursor diamine putrescine are aliphatic cations which are present in millimolar concentrations in all mammalian cells (1). They are aliphatic polycations which are positively charged at physiological pH and are among the most cationic small molecules within the cell (2). Unlike the point charges of Mg^{2+} and Ca^{2+}, the charge is distributed along the flexible carbon chains which enable the polyamines uniquely to bridge critical distances and thus allow specific interactions with polyanionic macromolecules such as DNA (3). The most obvious specific characteristic of the polyamines is their polybasic character which gives them a much higher affinity for acidic constituents than that exhibited by Na^+, K^+, Mg^{2+}, Ca^{2+}, or monoamines; this polybasic character is most pronounced with spermine because of its four positive groups. The polyamines are required for optimal growth of almost all cell types and increased biosynthesis is necessary for the traverse of a cell through the cell cycle. In contrast, depletion of polyamine content through inhibition of biosynthesis or via mutation of the key enzymes involved in their production results in significant inhibition of cell growth (4).

The cellular DNA replication factors include two DNA polymerases, DNA polymerase α (pol α) and δ (pol δ) and a number of novel DNA replication proteins, including replication protein A (RPA), replication factor C (RF-C) and the proliferating cell nuclear antigen (PCNA) (5). The pol α-primase complex is responsible for the synthesis of the first DNA segment, which could be considered the first lagging strand Okazaki fragment but is subsequently utilized as the primer for the initiation of leading strand synthesis by pol δ (5,6). Pol δ is shown carrying out elongation of the leading daughter strand in the presence of RPA and in conjunction with the protein factors PCNA and activator 1 (A1), also identified as RF-C. RPA mediates the unwinding of SV40 origin-containing DNA in the presence of SV40 T-Ag and topoisomerase. The interaction of SV40 T-Ag with DNA pol α-primase complex is required for the initiation of SV40 DNA replication (7-9). Once replication initiates, the elongation phase continues by DNA pol δ.

Several studies have demonstrated that DNA synthesis is inhibited in cells depleted of polyamines (2,10). The key enzyme in polyamine formation is ornithine decarboxylase, required for synthesis of putrescine, which serves as the precursor for the two higher polyamines, spermidine and spermine. The CHO P22 cells with no detectable ornithine decarboxylase activity grow well in the absence of serum if polyamines are available, but without added polyamines they gradually cease growing. DNA synthesis in CHO P22 cells and in primary human embryo lung fibroblasts both during polyamine starvation and under normal conditions have been studied. [^{32}P]orthophosphate labeled two sizes of DNA, a low

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Key words: polyamine, DNA replication, replication protein A, topoisomerase, DNA polymerase

molecular weight DNA corresponding to a marker of ~450 bp and a high molecular weight DNA, were founded. The ^{32}P-label appeared first in the short DNA species. After a pulse-chase the small size DNA gradually lost the radioactivity in normal cells, while the large DNA gained label, but in polyamine-starved cells the DNA fragments lost their label without incorporation of the radioactivity into the high molecular weight DNA. It is suggested that polyamines play an important role in the ligation of Okazaki fragments and the failure to ligate Okzaki fragments into long DNA chains might be the primary cause for the cessation of the cellular growth after polyamine depletion (10).

DNA pol α-primase, on the lagging strand, synthesizes short RNA-primed DNA fragments in the presence of RPA. Joining of these Okazaki fragments to form a completed daughter strand requires the action of a 5'→3'-exonuclease and RNase H to remove the RNA primers, filling in the gaps by a polymerase, and sealing the nicks by DNA ligase. In this study, the roles of polyamines in the DNA replication were examined using SV40 DNA replication system *in vitro*. We suggest that the major inhibition of SV40 DNA replication may be due to the inhibition of pol α-primase activity, and possible roles for polyamines in initiation process are discussed.

Materials and methods

Proteins, cell extracts, and DNA. SV40 origin-containing circular duplex DNA (pUC-ori$^+$), SV40 T-Ag, topo I, human DNA pol α-primase, RPA, and HeLa extract were prepared as described previously (11,12).

In vitro SV40 DNA replication assay. The reactions were carried out as described previously (7). In brief, the reaction mixtures (40 μl) included 40 mM creatine phosphate/di-Tris salt (pH 7.7), 1 μg of creatine kinase, 7 mM $MgCl_2$, 0.5 mM DTT, 4 mM ATP, 200 μM UTP, GTP, and CTP, 100 μM dATP, dGTP, and dCTP, 25 μM [^{3}H]dTTP (300 cpm/pmol), 0.6 μg of SV40 T-Ag, 0.23 μg of pUC-ori$^+$, HeLa extracts, and polyamines as indicated. The reactions ran at 37˚C for 2 h, after which the acid-insoluble radioactivity was measured. Replication products were analyzed using [α-^{32}P]dATP (30,000 cpm/pmol) instead of [^{3}H]dTTP in these reactions. Replication products in the reaction mixture were analyzed by electrophoretically separating the isolated DNA in a 1.0% agarose gel overnight at 42 V. The gel was subsequently dried and exposed to X-ray film.

Topoisomerase assay. Topoisomerase activity was measured by the relaxation of superhelical plasmid DNA (13). The 20 μl assay mixture contained 50 mM Tris-HCl (pH 7.5), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, bovine serum albumin (30 μg/ml), pSA (20 μg/ml), and various amount of the enzyme. After 30 min at 30˚C, the reactions were stopped by the addition of 5 μl of 5% $NaDodSO_4$/25% (wt/vol) Ficoll 400 (Pharmacia) containing 0.25 mg of bromophenol blue per ml. The electrophoresis and photography were done as described. One unit of activity is the amount of topoisomerase that relaxes half of the superhelical plasmid DNA under these conditions.

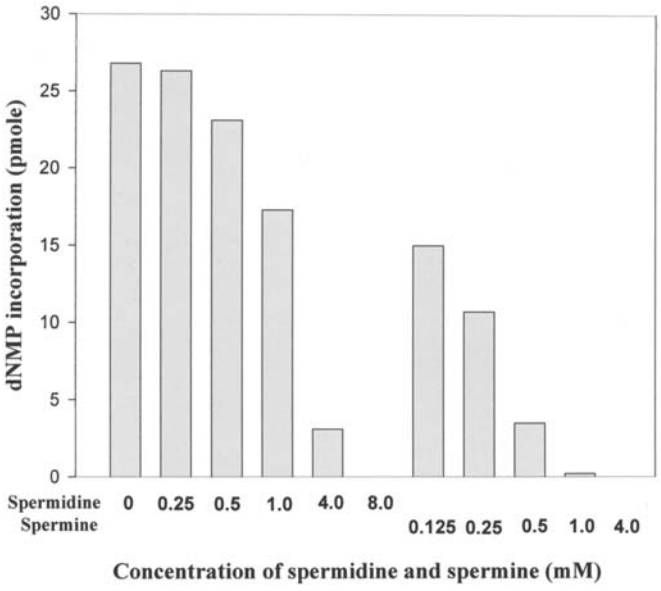

Figure 1. The effect of polyamines on SV40 DNA replication *in vitro*. Replication reaction comprised SV40 origin-containing DNA (pUC-ori$^+$), SV40 T-Ag, HeLa cytosolic extract (100 μg), [^{3}H]dTTP, and the indicated amounts of polyamines. Reaction mixtures were incubated for 2 h at 37˚C, and the reaction products examined for acid-insoluble radioactivity.

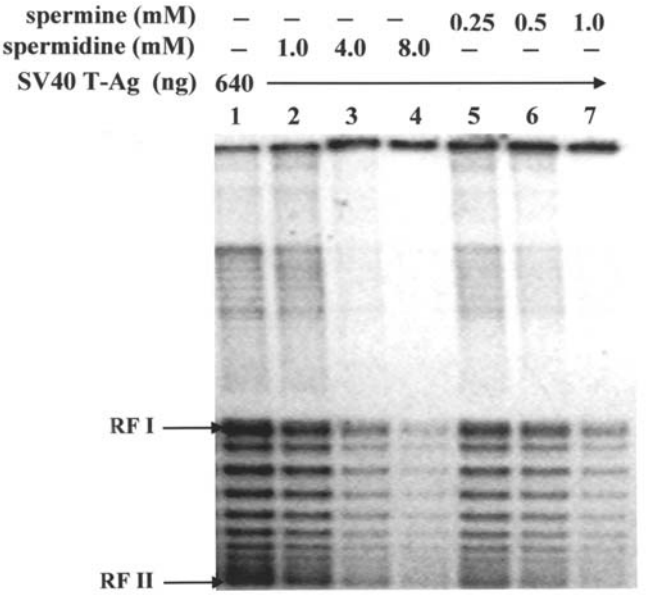

Figure 2. The effect of polyamines on SV40 DNA replication *in vitro*. Replication reaction comprised SV40 origin-containing DNA (pUC-ori$^+$), SV40 T-Ag, HeLa cytosolic extract (100 μg), [α-^{32}P]dATP, and the indicated amounts of polyamines. Reaction mixtures were incubated for 2 h at 37˚C, and the reaction products were isolated and separated by 1% agarose gel electrophoresis (Tris-borate-EDTA buffer).

ssDNA binding assay. The ssDNA binding activity was measured according to the published procedures (14). The reaction mixtures (20 μl) contained 50 mM Hepes-KOH (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 10% glycerol, 250 fmol of 5'-^{32}P-labeled $(dT)_{50}$ (1,200 cpm/fmol), and the incubated amounts of RPA. After incubating the reaction mixtures for 15 min at 25˚C, the DNA-protein

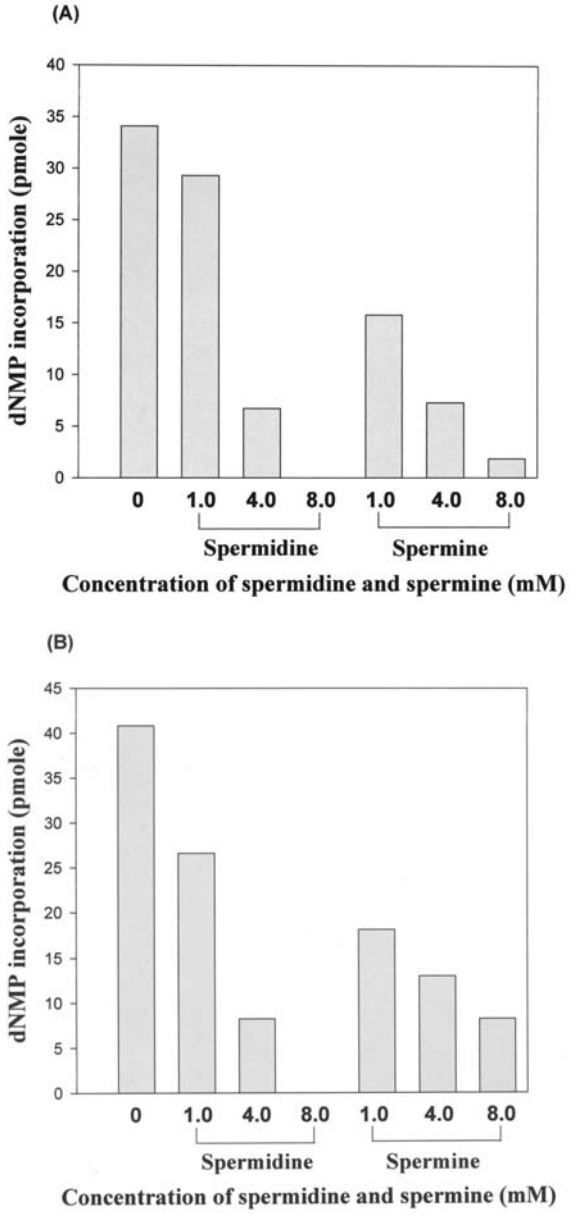

Figure 3. Polyamines inhibited the same initiation stage as elongation stage of SV40 DNA replication *in vitro*. Polyamines were added to reaction mixture before (A) or after (B) the preincubation in the presence of ATP at 37˚C for 30 min. [^{3}H]dNTPs were then added, and the mixture was incubated for 2 h at 37˚C, and the reaction products examined for acid-insoluble radioactivity. All the conditions were the same as those described in the legend for Fig. 2.

complexes were electrophoretically separated in a 5% polyacrylamide gel in 0.5X TBE buffer at 12 V/cm.

Polymerase assay. Polymerase activity was examined out as described previously (15) with the following modifications: the reaction mixtures (30 μl) included 40 mM creatine phosphate/di-Tris salt, pH 7.7, creatine kinase 1.0 μg, 7 mM $MgCl_2$, 1.0 mM DTT, BSA 6 μg, 4 mM ATP, [^{3}H]-dTTP 33 μM, 0.1 μg poly(dA)$_{4500}$:oligo(dT)$_{25}$, DNA pol α-primase. The reactions ran at 37˚C for 2 h, after which the acid-insoluble radioactivity was measured.

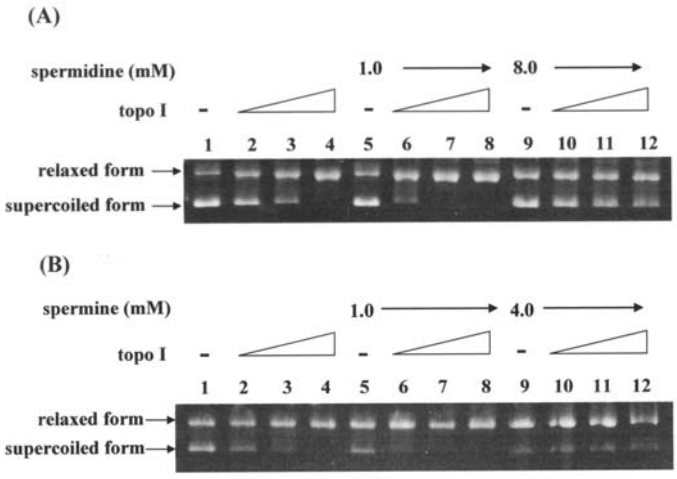

Figure 4. Polyamines inhibited topo I catalytic activity. Topoisomerase activity was measured by the relaxation of superhelical plasmid DNA. The assay mixture (20 μl) contained pSA (20 μg/ml), topo I, and various amounts of the spermidine (A) and spermine (B). After 30 min at 30˚C, the reactions were stopped by the addition of 5 μl of stop solution. The samples were then loaded onto the agarose gel (0.8%) for electrophoresis and photography was done.

Results

Polyamines inhibited SV40 DNA replication in vitro. The roles of polyamines on DNA replication were examined using SV40 DNA replication system *in vitro* (Fig. 1). Polyamines showed inhibitory effects on DNA replication in SV40 DNA replication system. Spermidine showed inhibitory effect with 88% inhibition at 4.0 mM. Spermine showed inhibitory effect with 87% inhibition at 0.5 mM. Spermine showed a more inhibitory effect than spermidine. Agarose gel electrophoresis (Fig. 2) shows that the replication products include RF I, RF II (circular duplex DNA containing at least one single-strand break), intervening topoisomers, and discrete slower-migrating species. In addition, increasing polyamines quantitatively inhibited SV40 DNA replication. Three factors, SV40 T-Ag, RPA, and pol α-primase complex, are essential for the initiation process. In the presence of topoisomerase, SV40 T-Ag will continue to unwind the DNA to form a highly unwound DNA. DNA synthesis with three factors and topoisomerase can be quite extensive. We questioned which stage of DNA replication is inhibited by polyamines.

Which stage of DNA replication was inhibited by polyamines? We examined whether the inhibitory effect of polyamines on SV40 DNA replication *in vitro* occurs at the level of initiation or elongation. Formation of a DNA-protein complex is a prerequisite for initiation of SV40 DNA replication. This complex is formed in the absence of dNTPs at 37˚C and is dependent on the replication origin, SV40 T-Ag, ATP, and host proteins. Subsequent DNA synthesis in the presence of dNTPs occurs at either 25˚C or 37˚C. Under standard reaction conditions, DNA replication displays biphasic kinetics. During the first phase, which lasts ~10-20 min, minimal or no DNA synthesis is observed. During the second phase, the rate of DNA synthesis increases rapidly to a relatively constant level that is maintained for at least 2 h.

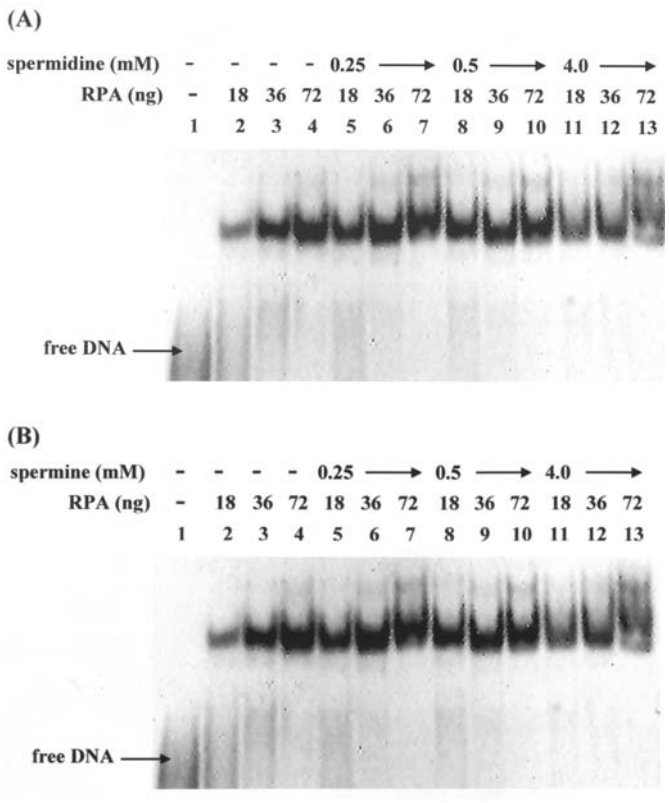

Figure 5. The effect of polyamines on RPA's ssDNA binding activity. Indicated amounts of either human RPA, or a mixture of both RPA and spermidine (A) and spermine (B) were combined with ^{32}P-labeled $(dT)_{50}$ and incubated at 25˚C for 15 min. The protein-DNA complexes were then separated from unbound DNA by 5% polyacrylamide (acrylamide : bisacrylamide = 29:1) gel electrophoresis.

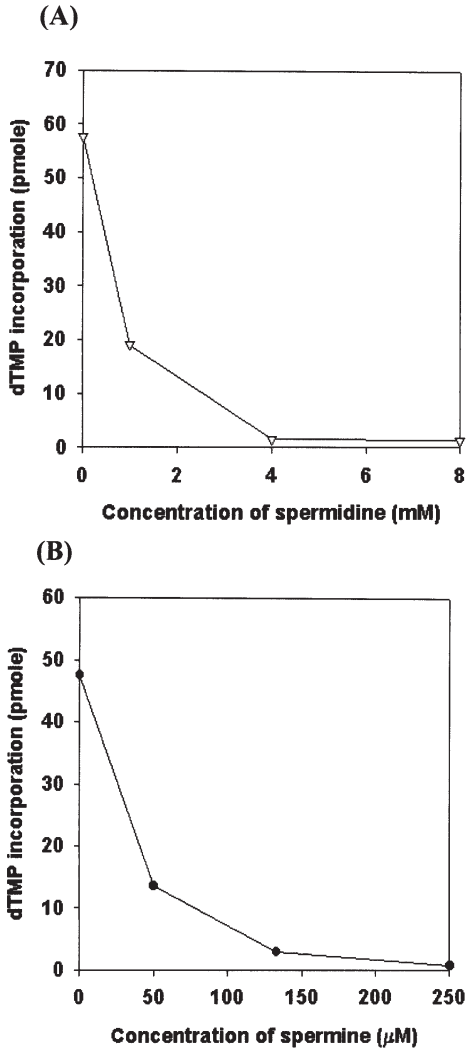

Figure 6. The effect of polyamines on pol α-primase activity. (A) Indicated amounts of spermidine were added to the reaction mixture. (B) Indicated amounts of spermine were added to the reaction mixture, which included 0.1 unit of human pol α-primase complex, 4 mM ATP, 1 mM [^{3}H]dTTP, and 0.1 μg of poly$(dA)_{4500}$:oligo$(dT)_{12\text{-}18}$. Incubation ran at 37˚C for 30 min, followed by measurement of acid-insoluble radioactivity.

The initial lag in DNA synthesis can be eliminated if the complete reaction mixture is preincubated at 37˚C in the absence of deoxynucleoside triphosphates. As shown in Fig. 3A and B, in the presence of polyamines, the replication activities were inhibited if added to reaction mixture before the preincubation in the presence of ATP (Fig. 3A), and also inhibited if added after the preincubation (Fig. 3B). Therefore, it is possible that polyamines may inhibit the initiation or elongation stage of DNA replication at nearly the same degree.

Polyamines inhibited topoisomerase I activity. We showed that it is not clear at which stage the polyamine inhibition occurred. Based on their properties, we suggest that polyamines might inhibit either an origin-binding protein or some other proteins required to establish a replication fork during the initiation reaction. To address this possibility, we asked whether polyamines inhibit topo I activity. By forming transient DNA single-strand breaks and acting as DNA strand transferase, topo I plays key roles in DNA replication, transcription, and recombination. The inhibitory effects of polyamines on the catalytic activity of topo I are shown in Fig. 4A and B. The plasmid DNA was in the superhelical form (lane 1), and topo I relaxed the supercoiled DNA (lane 2). Spermidine inhibited the DNA cleavage by topo I at 8.0 mM, but stimulated its activity at 1.0 mM. Spermine also inhibited topo I activity at 4.0 mM, but stimulated at 1.0 mM.

The activity of RPA was slightly affected by polyamines. In replication, RPA mediates unwinding of SV40 origin-containing DNA in the presence of SV40 T-Ag and topoisomerase. It interacts with SV40 T-Ag and the DNA pol α-primase complex, which is necessary for the initiation of SV40 DNA replication. We examined whether polyamines affect ssDNA-binding activity of RPA. As shown in Fig. 5A and B, RPA formed stable complexes with oligo$(dT)_{50}$, which appeared as two distinct bands in the polyacrylamide gel. The ssDNA binding activity of RPA was slightly affected by polyamines.

Polyamines significantly inhibited DNA pol α-primase activity. As described above, DNA pol α-primase complex is necessary for the initiation of SV40 DNA replication. To further investigate the inhibitory effect of polyamines in replication, we tested polyamines for inhibition of pol α activity to see whether their inhibitory effects on DNA replication correlate

with pol α activity. As shown in Fig. 6A and B, spermidine completely inhibited pol α-primase activity (Fig. 6A) at 4 mM, and spermine at 133 μM (Fig. 6B). The above results indicate that major inhibition of SV40 DNA replication by polyamines may be due to the inhibition of pol α-primase activity. Herein, we cannot rule out the possibility that polyamines inhibit the activity of DNA pol α, because it is essential for the elongation process.

Discussion

In *Escherichia coli* it has been suggested that polyamines may function in an unwinding protein and interact directly with the DNA (16). Polyamines can influence the binding of the pol α to the 3'-hydroxyl primer in order to decrease the reaction velocity. Polyamine has also been shown to increase the rate of movement of the replication fork and hence increase the rate of chain elongation in a polyamine-deficient strain of *E. coli*.

The exact mode of action of the polyamines on DNA replication is unclear. In this study, the role of polyamines in DNA replication was examined using the SV40 DNA replication system *in vitro*. We found that polyamines showed inhibitory effect on DNA replication *in vitro*. However, it is not clear at which stage of DNA replication the inhibition occurs.

In SV40 DNA replication, three factors, SV40 T-Ag, RPA, and pol α-primase complex, are essential for the initiation process (17,18). In the presence of topoisomerase, SV40 T-Ag will continue to unwind the DNA to form a highly unwound DNA (19). DNA synthesis with three factors and topo-isomerase can be quite extensive (20). We have suggested that polyamines might interfere with some molecules that are required to establish replication forks during the initiation reaction (21). To address this possibility, we asked whether polyamines inhibit topo I, the ssDNA binding activity of RPA, and pol α-primase activity. Polyamines inhibited the DNA cleavage by topo I. Spermidine inhibited the DNA cleavage by topo I at 8.0 mM, but stimulated its activity at 1.0 mM. Spermine also showed inhibitory effect on topo I activity at 4.0 mM, but stimulation at 1.0 mM. Our results suggest that the ratio of DNA to polyamine may be an important factor in determining whether the topo I reaction is stimulated or inhibited.

The ssDNA binding activity of RPA was slightly affected by polyamines. We thought that the inhibitory effects of poly-amines in DNA replication might not correlate with the activity of RPA. On the other hand, spermidine, at 4 mM, reduced pol α-primase activity, and spermine significantly reduced at 133 μM. The above results indicate that major inhibition of SV40 DNA replication by polyamines may be due to the inhibition of pol α-primase activity. However, we cannot rule out the possibility that polyamines inhibit the activity of DNA pol δ, because it is essential for the elongation process.

Based on their properties, we suggest that polyamines inhibit some molecules that are required to establish replication forks during the initiation reaction or inhibit synthesis of Okazaki DNA fragment through inhibition of pol α-primase activity.

Acknowledgements

This study was supported by the 2004 Inje University research grant.

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