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Effects on the Initiation of Simian Virus 40 DNA Replication by Antisense RNA

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Abstract: When DNA replication of simian virus 40 (SV40) is initiated on the replication origin, the regions containing the initiation sites of DNA primase, which participates in the transient RNA primer synthesis for formation of Okazaki fragments in the lagging strand, were chosen as the target sites of antisense RNA for studies of the inhibition of SV40 DNA replication. Four recombinant transcription vectors, pUC-PrI, pUC-PrII, pGEM-PrBS, and pGEM-PrSN, coding antisense RNA, were constructed. Four antisense RNAs (named as I, II, BS, and SN) having the size of 18, 19, 58, and 123 nts, respectively, were made from the transcription vectors by *in vitro* transcription. And then, antisense RNA in the concentration of 2 μM were added to COS cells transfected with pATSV-W which is a recombinant plasmid containing the SV40 origin of replication. The inhibitory extent of DNA replication was measured by *DpnI* resistance and was confirmed by measurement of transient RNA primer synthesis. The result shows that six combinations of antisense RNA (I, II, BS, SN, I+SN, and BS+SN) lead to the inhibition of SV40 DNA replication by up to 85%.

Key words: antisense RNA, inhibition, RNA primer, SV40 DNA replication.

It is known that antisense RNA inhibits specifically gene expression through base-paring with the target DNA or RNA in prokaryotes and eukaryotes (Green *et al.*, 1986; Holt *et al.*, 1986; Wormington, 1986; Delauney *et al.*, 1988; Lichtenstein, 1988; van der Krol *et al.*, 1988; Walder, 1988; Daugherty *et al.*, 1989; Skeiky and Iatrou, 1990; Takayama and Inouye, 1990; Henze, 1991). In the case of the inhibition of replication, the example of natural antisense RNA is the inhibition of formation of Col E1 RNA primer by plasmid-specific small RNA (Tomizawa *et al.*, 1981; Tomizawa, 1990a and 1990b). Most of the inhibition of replication by antisense RNA was examined in viral DNA or RNA (Chang and Stoltzfus, 1987; Jennings and Molloy, 1987; Miroshnichenko *et al.*, 1989; Shibahara *et al.*, 1989; von Ruden and Gilboa, 1989; Jonhi *et al.*, 1991; Rittner and Sczakiel, 1991; Sczakiel and Pawlita, 1991). But, most of these involved experiments by antisense RNA expressed in host cells, except the inhibition of human immuno-deficiency virus replication by synthetic oligomer-RNA derivatives (Shibahara *et al.*, 1989). These examples contain Adenovirus 5 (Miroshnichenko *et al.*, 1989), human immuno-deficiency virus (Shibahara *et al.*, 1989; von Ruden and Gilboa, 1989; Jonhi *et al.*, 1991; Rittner and Sczakiel, 1991), simian virus 40 (Jennings and Molloy, 1987) and Rous sarcoma virus (Chang and Stoltzfus, 1987).

Simian virus 40 (SV40) DNA replication (Kelly, 1988) in permissive cell depends on host cellular factors or enzymes that are required for the replication machinery except the origin of replication (Deb *et al.*, 1986; Guo *et al.*, 1989; Parsons *et al.*, 1990) and T antigen (Ag) (Parsons *et al.*, 1991). SV40 DNA forms minichromosome which is similar to the chromatin structure of chromosome in nucleus. Because the replication of SV 40 DNA is similar to the mechanism of chromosomal DNA replication, SV40 DNA has been used in many investigations for research of chromosomal DNA replication in eucaryotes.

In the present study, we have chosen the regions containing the initiation sites of DNA primase which participates in the transient RNA primer formation for the synthesis of Okazaki fragments on the lagging strand in the initiation of SV40 DNA replication, as target sites of antisense RNA (Fig. 1). We have also investigated the effect of antisense RNA on SV40 DNA replication and its result was confirmed by the inhibitory effect of antisense RNA on the synthesis of transient RNA primer.

Materials and Methods

Cell culture

COS cells are an African green monkey kidney cell

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line transformed with an origin-defective mutant of SV 40 and express the SV40 large and small T antigens constitutively (Gerard and Gluzman, 1985). These cells were cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) at 37℃ in 5% CO_2 atmosphere.

Plasmids and bacterial strains

The plasmid used for replication assay was the plasmid pATSV-W (Kim and Kang, 1989) containing the SV40 origin of replication. The plasmids, pUC18 and pGEM-3Z, were used in the construction of transcription vectors for preparation of antisense RNA. The host for preparation of the plasmid pATSV-W was *E. coli* strain HB101 [*supE*44 *hsdS*20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1]. As the host for antisense transcription vector, *E. coli* strain XL1-blue [*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ *F'*[*proAB*$^+$ *lacI*^{q} *lacZ* Δ*M*15 *Tn*10(*tet*^{r})] was used.

***In vitro* transcription**

For run-off transcription, the antisense RNA transcription vectors pUC-PrI, pUC-PrII, pGEM-PrBS, and pGEM-PrSN were digested with the restriction enzymes *Xba*I, *Sma*I, *Bam*HI, and *Eco*RI, respectively. The transcription reaction volume was 100 μl and contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 5 mM NaCl, 40 mM DTT, 0.5 mM each ATP, CTP, GTP, and UTP, 5 μg of linearized template DNA, 40 U of SP6 RNA polymerase, and 1 U of ribonuclease inhibitor per μl. This mixture was incubated for 1 h at 37℃ or 42℃, and after 10 U of RNA polymerase were added to the reaction mixture and the incubation was continued for 1 h. To remove DNA template, the reaction mixture was treated with 5 U of RNase-free DNase I (Boehringer Mannheim) for 15 min at 37℃ and treated with phenol/chloroform to remove proteins and precipitated with 2.5 volumes of ethanol. The quantity and purity of transcript was measured in the absorbance at 260 nm and 280 nm. These products were used as antisense RNAs.

Transfection

COS cells of 60~70% confluent (1×10^6 cells) on 60 mm dish were transfected with 1 μg of pATSV-W by transfection method using DEAE-dextran (Lopata *et al.*, 1984) and then were incubated in DMEM containing 2% FBS for 48 h at 37℃ in 5% CO_2 atmosphere.

DNA replication assay

Antisense RNAs were added to the media of COS cells transfected with pATSV-W in the concentration of 2 μM. After 48 h, pATSV-W was extracted from pATSV-W-transfected COS cells by Hirt's method (Hirt, 1967). The isolated DNA was digested with the restriction enzyme, *Dpn*I, to remove input pATSV-W and also was digested with the restriction enzyme, *Pst*I, to linearize progeny pATSV-W. This digested DNA was electrophoresed on 1% agarose gel and then transferred to nitrocellulose filter paper. [^{32}P]probe was prepared by nick-translation of pATSV-W in the presence of [α-^{32}P]dATP (sp. act.=6,000 Ci/mmol). After hybridization, the filter paper was washed and exposed to X-ray film (Hyper-MP film, Amersham). The band intensity of the autoradiogram was measured by a densitometric scanner.

***In vivo* RNA priming assay**

COS cells transfected with pATSV-W were incubated with [5,6-^{3}H]uridine (100 μCi/ml, sp. act.=44 Ci/mmol) for 4 h at 37℃, at 28 h after transfection. 2 μM antisense RNA was added to the culture after transfection. And then, low-molecular weight of plasmid DNA was extracted from the transfected COS cells by Hirt's method and were centrifuged to equilibrate in CsCl containing 200 μg of ethidium bromide per ml on a Beckman Ti80 rotor at 270,000×*g* for 24 h at 20℃ (CsCl, ρ=1.55 g/ml). The fractions were collected from the bottom and was counted by a liquid scintillation counter (Beckman Palo Alto, USA).

Results

Construction of *in vitro* transcription vectors

pUC-PrI and pUC-PrII (Fig. 2A): *In vitro* transcription vectors constructed for preparation of antisense RNAs correspond to nucleotide position (np) 5,208~5,223 and np 44~60 containing primase initiation sites (Fig. 1). The complementary 40-mers (a and b) and 39-mers (a and b) that have a SP6 promoter region in the front of base sequence of np 5,208~5,223 and np 44~60, and also have restriction enzyme sites for cloning in 5'-end and 3'-end, respectively, were synthesized in a DNA synthesizer (Beckman). The sequence of oligomers is as follows;

For pUC-PrI
40-mer(a):
5'-AATTCATTTAGGAGACACTATACCTCACTACTTCTGGAAT-3'
40-mer(b):
5'-CTAGATTCCAGAAGTAGTGAGGTATAGTGTCACCTAAATG-3'
For pUC-PrII
39-mer(a):
5'-AATTCATTTAGGTGACACTATATTCCGCCCATTCTCCGC-3'
39-mer(b):
5'-CCGGGCGGAGAATGGGCGGAATATAGTGTCACCTAAATG-3'

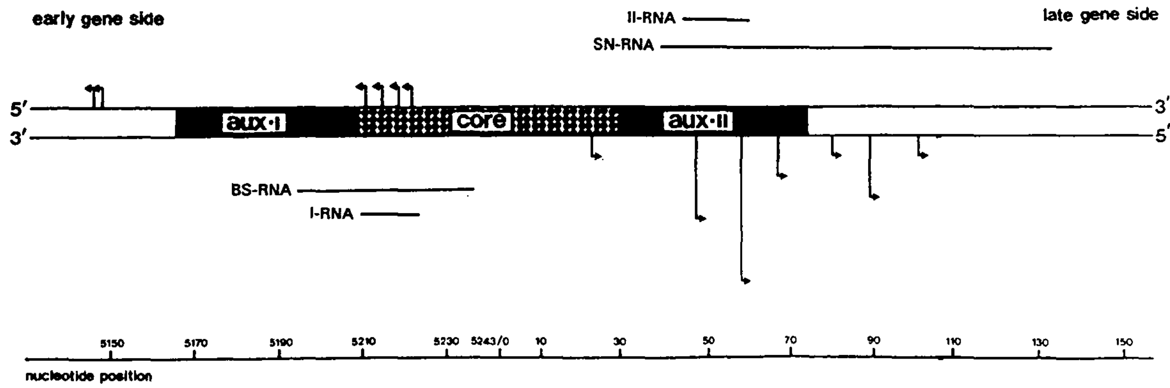

Fig. 1. The SV40 origin of replication and the target sites of antisense RNAs. The SV40 origin of replication is constructed with the ori-core region, auxiliary region I (aux-I) and auxiliary region II (aux-II). Ori-core region is essential to SV40 DNA replication and auxiliary regions are a kind of enhancer for SV40 DNA replication. As known by Tseng *et al.* (Tseng and Ahlem, 1984; Tseng and Prussak, 1989), the primase initiation sites consist of the sites on the early gene side and on the late gene side. I and BS are the same sequences as nucleotide position (np) 5,208~5,225 and np 5,193~5,239, respectively, of the strand containing primase initiation sites. Thus, the opposite strand is the target site. Also, II and SN are identical with the sequence of strand containing primase initiation sites of np 60~44 region and np 133~38 region, respectively, and the opposite region is the target site. The length of arrow is represented the frequency of the primase initiation, and the direction of arrowhead is indicated the orientation.

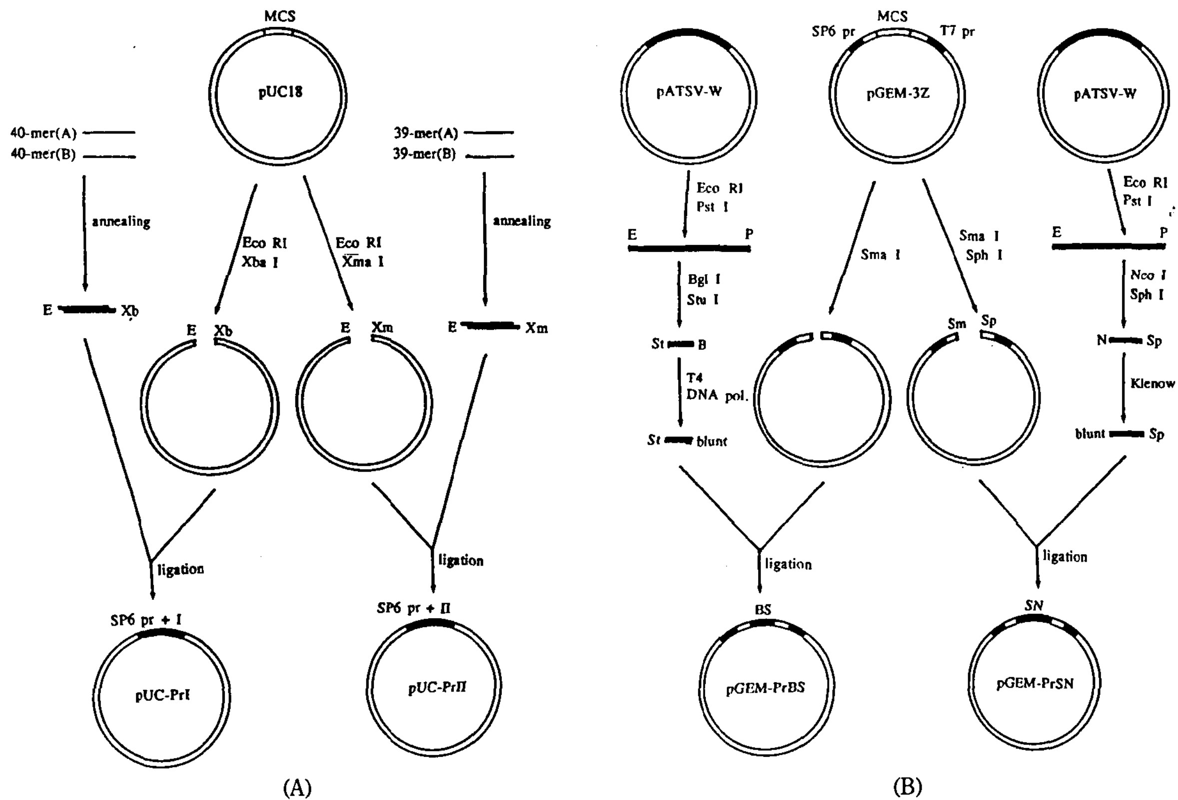

Fig. 2. The construction of *in vitro* transcription vector. (A) pUC-PrI and pUC-PrII. The complementary 40-mers (a and b) and 39-mers (a and b) that have a SP6 promoter region in the front of nucleotide position (np) 5,208~5,223 and np 44-60, and that have restriction enzymes for cloning in 5'-end and 3'-end, respectively, were synthesized. The annealed 40-mers and 39-mers were cloned to *EcoRI-XbaI* site and *EcoRI-XmaI* site of multi-cloning sites of the plasmid pUC18, respectively. (B) pGEM-PrBS was constructed as follows: *StuI-BglI* (blunt-ended by a exoactivity of T4 DNA polymerase) 52 bp fragment that corresponds to np 5,193~5,239 near SV40 origin region was cloned to *EcoRI* (filled-in by klenow fragment)-*SmaI* site in MCS of the plasmid pGEM-3Z. pGEM-PrSN was constructed as follows: *NcoI* (filled-in by Klenow fragment)-*SphI* 90 bp fragment that corresponds to *SmaI-SphI* site in multi-cloning sites of the plasmid pGEM-3Z.

To phosphorylate of oligomer in 5′-end, it was incubated in 50 μl reactions containing 2 μg of ODN, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM ATP, and 16 U of T4 polynucleotide kinase for 30 min at 37℃. The phosphorylated oligomers were annealed by the following subsequent reactions; for 2 min at 85℃, 15 min at 65℃, 15 min at 37℃, 15 min at room temperature, and 15 min on ice. The annealed 40-mers containing *Eco*RI and *Xba*I and 39-mers containing *Eco*RI and *Xma*I sites at 5′-end and 3′-end, respectively, were ligated to *Eco*RI-*Xba*I site and *Eco*RI-*Xma*I site of multi-cloning sites of the plasmid pUC18, respectively, and transformed to *E. coli* XL1 blue and selected by α-complementation (Ullman *et al.*, 1967) in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropylthio-β-D-galactoside (IPTG).

pGEM-PrBS (Fig. 2B): The *Stu*I-*Bgl*I fragment of 52 bp that corresponds to np 5,193~5,239 near the SV40 origin was prepared. The *Bgl*I site was blunt-ended by an exonuclease activity of T4 DNA polymerase. This fragment was ligated to *Eco*RI (blunt-ended by klenow fragment)-*Sma*I site in multi-cloning sites of the plasmid pGEM-3Z.

pGEM-PrSN (Fig. 2B): The *Nco*I-*Sph*I fragment of 90 bp that corresponds to np 133~38 near the SV40 origin was prepared. *Nco*I site was filled-in by Klenow frgment. This fragment was ligated to *Sma*I-*Sph*I site in multi-cloning sites of the plasmid pGEM-3Z.

Preparation of antisense RNA

RNA polymerase which is encoded by bacteriophage SP6 or T7 is an enzyme that efficiently synthesizes a defined RNA sequence from the cloned DNA. Antisense RNA was made from four transcription vectors by SP6 RNA polymerase (Fig. 3). To prepare the homogenous transcript, the reaction of transcription was done after restriction enzyme treatment of template DNA. The RNA transcripts from pUC-PrI, pUC-PrII, pGEM-PrBS, and pGEM-PrSN were named as antisense RNA I, II, BS, and SN, respectively. The length of I and II is 18 nts and 19 nts, respectively, and the transcription yield was more efficient at 42℃ rather than at 37℃ (data not shown). The transcription reaction for BS and SN was done at 37℃. Under these conditions, approximately 10 μg of antisense RNA per μg of template DNA was made. These transcripts were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and confirmed by electrophoresis on the denaturing acrylamide gel and autoradiography.

Stability of antisense RNA in COS cells

After the treatment with 2 μM [^{32}P]RNA, cells were harvested at the time intervals of 0.5, 1, 2, 3, 5, 8,

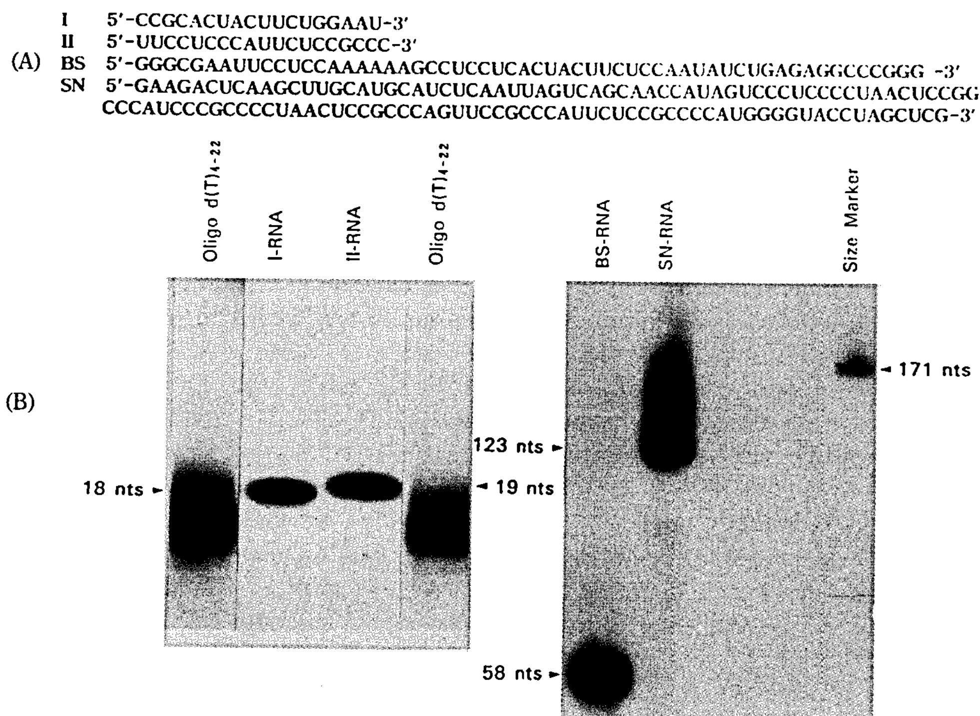

Fig. 3. The sequence of antisense RNA and its autoradiogram. (A) The RNA transcript size from pUC-PrI, pUC-PrII, pGEM-PrBS, and pGEM-PrSN is 18, 19, 58, and 123 nts, respectively. These transcripts were designated as antisense RNA I, II, BS, and SN, respectively. (B) The RNA transcripts were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and electrophoresed on the denaturing 8% (for BS and SN) and 20% (for I and II) acrylamide gel and autoradiographed.

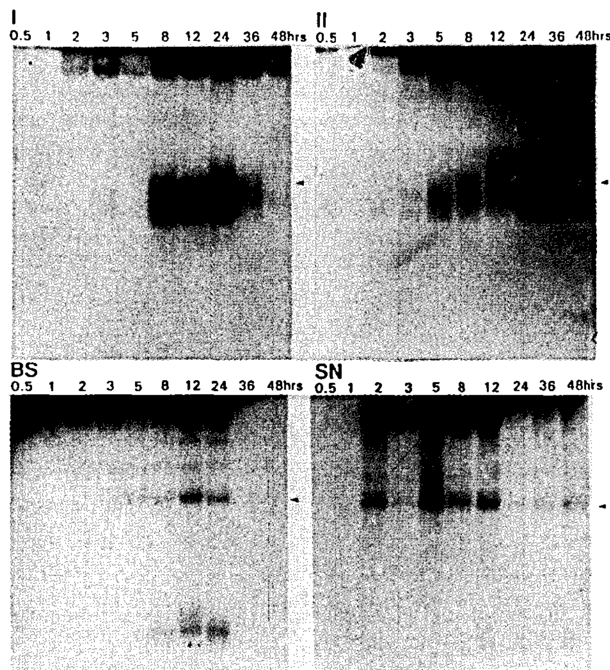

Fig. 4. Stability of antisense RNA in COS cells. 9.5×10^3 cells per cm^2 well were incubated with 2 μM RNA for 0.5, 1, 2, 3, 5, 8, 12, 24, 36, and 48 h, respectively. [^{32}P]RNA was isolated from COS cells and electrophoresed on the denaturing 20% (for I and II) and 8% (for BS and SN) acrylamide gel and autoradiographed. It shows that I and II were detected in the degraded state generally, and that BS and SN were maintained in the intact state to what extent. The arrowhead indicates the intact RNA.

12, 24, 36, and 48 h. [^{32}P]RNAs extracted from COS cells were electrophoresed on the denaturing acrylamide gel (I and II, 20% acrylamide gel; BS and SN, 8% acrylamide gel) and autoradiographed (Fig. 4). I and II was detected at the time zone 8-36 h but the intact form was few. In the case of BS, the uptake was increased to 24 h and thereafter decreased. In the case of SN uptake, the pattern of the increase and decrease was shown. It was reported that this phenomenon was observed on the uptake of oligomer by HL-60 cells (Wickstrom *et al.*, 1988). But the mechanism of these phenomena is unknown. Generally, BS and SN was maintained in the intact state but degraded considerably by the attack of several ribonucleases.

Antisense RNA is detected in nucleus

Because DNA replication event occurs in the nucleus, the detection of antisense RNA in the nucleus is important. Actually, BS was detected by 2.2% in the cell and 0.2% in the nucleus. Thus, 10.2% of BS in the cell was translocated to the nucleus. Similarly, SN was uptaken in the cell by 2.1% and detected in nucleus by 0.2%. Thus, 10.9% of SN in the cell was translocated to the nucleus (Table 1). It is assumed that this translocation of antisense RNA to the nucleus is mediated by nuclear membrane-bound proteins.

Table 1. Quantification of antisense RNA in nucleus

(A) BS-RNA

	cpm	RNA, μM	% Uptake
Input	2.45×10^5	2	–
Cell	5.28×10^3	4.30×10^2	2.15
Nucleus	5.32×10^2	4.33×10^2	0.22
Nucleus/cell	–	–	10.23

(B) SN-RNA

	cpm	RNA, μM	% Uptake
Input	1.04×10^5	2	–
Cell	2.22×10^3	4.25×10^2	2.12
Nucleus	2.44×10^2	4.68×10^2	0.23
Nucleus/cell	–	–	10.85

COS cells were incubated in DMEM containing 2% FBS in the treatment with [^{32}P]BS or [^{32}P]SN (2 μM) for 12 h at 37°C. Nucleus was extracted from cell and the amount of antisense RNA in nucleus was measured by a liquid scintilation counter.

SV40 DNA replication is inhibited by antisense RNA

Input plasmid, pATSV-W which was replicated in the dam$^+$ *E. coli* strain HB101, was methylated at *Mbo*I site and is sensitive to *Dpn*I. However, progeny pATSV-W which was replicated in mammalian cells is unmethylated because mammalian cells lacks methylase. Thus, DNA samples were digested with *Dpn*I to distinguish between the *Dpn*I-sensitive input DNA and the *Dpn*I-resistant newly replicated progeny DNA. The effect of antisense RNA on SV40 DNA replication was measured as the extent to *Dpn*I-resistance of progeny DNA.

Antisense RNAs were added to the culture of COS cells transfected with pATSV-W. From the result of DNA replication assay, it was shown that SV40 DNA replication was inhibited by antisense RNA (Fig. 5). In the treatment with a kind of antisense RNA, SV40 DNA replication was inhibited as follows: I inhibited by 10%; II, 40%; BS, 10%; and SN, 80%. In the treatment with two kinds of antisense RNA, SV40 DNA replication was inhibited by 85% by I+SN and 65% by BS+SN: exceptionally, by I+II or II+BS, DNA replication was increased by 10 and 20%, respectively. In the treatment with random RNA without sequence specificity to the target of antisense RNA, it was shown that random RNA with the length of 60 nucleotides have no an effect on DNA replication and RNA primer synthesis (data not shown).

Synthesis of RNA primer is inhibited by antisense RNA

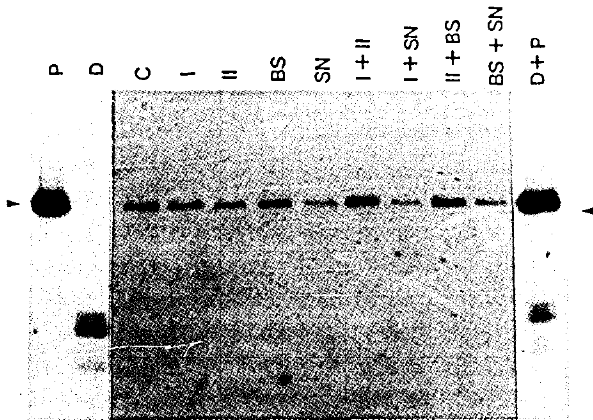

Fig. 5. Replication assay by DpnI resistance. Duplicated 60 mm dishes of COS cells were transfected with 1 μg of pATSV-W in the treatment with antisense RNAs. The concentration of antisense RNA used was 2 μM. After 48 h, low molecular weight of plasmid DNA was isolated, digested with DpnI to remove the input DNA, and digested with *Pst*I to linearize the progeny DNA, and was analyzed by 1% agarose gel electrophoresis and Southern-blot hybridization with nick-translated pATSV-W using [γ-^{32}P]dATP. C, pATSV-W *Pst*I (w/o antisense RNA treatment as control); P, pATSV-W *Pst*I; D, pATSV-W *Dpn*I; D+P, pATSV-W *Pst*I+pATSV-W *Dpn*I.

Table 2. Effect of antisense RNA on RNA primer synthesis

Antisense RNA	% Primer synthesis		
	I-form	RI-form	Average
G (control)	100.0	100.0	100.0
I	83.9	86.4	85.1
II	90.9	83.7	87.3
BS	97.3	100.3	98.8
SN	16.2	14.4	15.3
I+II	85.9	87.9	86.9
I+SN	10.9	25.0	18.0
II+BS	109.6	101.7	105.6
BS+SN	44.9	22.3	33.6

COS cells were labeled at 18 h after transfection for 4 h with [5,6-^{3}H]Turidine (sp. act.=44 Ci/mmol), 70 μCi/ml. Antisense RNA in the concentration of 2 μM were added to the media immediately after transfection. [^{3}H]pATSV-W was extracted by Hirt's method and fractionated by CsCl-EtBr equilibrium centrifugation (Ti80 rotor, 270,000×*g*, 24 h, 20°C, and CsCl=1.55 g/ml). The plasmid was resolved into two peaks as I (supercoil) form and RI (replicative intermediate) form. Basal level was measured by mock-transfection.

The band pattern of [^{3}H]pATSV-W on CsCl gradient was as follows: form I as supercoil and replicative intermediate form (RI form) as an open circle. This CsCl gradient was fractionated and counted by a liquid scintilation counter. The synthetic extent of RNA primer was shown in Table 2. In the treatment with a kind of antisense RNA, RNA primer synthesis was inhibited as follows: I, II, BS, and SN inhibited by 14.9%, 12.7%, 1.2%, and 84.7%, respectively. In the treatment with two kinds of antisense RNA, I+II, I+SN, and BS+SN inhibited by 13%, 82%, and 66.4%, respectively. However, it was shown that the inhibitory effect of antisense RNA I+II has a discrepancy with the enhanced effect on DNA replication. This phenomenon is not understood. And in the treatment with antisense RNA II+BS, the synthetic rate of RNA primer was increased by 5.6 % more than control and was similar to the result of DNA replication.

Discussion

From the studies related to replication known up to date, most has been done by addition of excessive antisense oligomer or RNA in comparison with target DNA or RNA molecules. For example, in the case of type A influenza virus (Zerial *et al.*, 1987), simian virus 40 (Birg *et al.*, 1990), human immuno-deficiency virus (Zamecnik *et al.*, 1978; Matsukura *et al.*, 1987; Agrawal *et al.*, 1988; Goodchild *et al.*, 1988; Sarin *et al.*, 1988), and Rous sarcoma virus (Zamecnik and Stephenson, 1978), it was treated to a maximum 300 μM of antisense molecules. In the treatment with excessive antisense molecules, it is possible to inhibit DNA replication in the nonspecific manner. Thus, the trapping effect of target DNA or RNA by antisense oligomer or RNA is indicated higher than actual effect. To reduce this possibility in the present study, 2 μM antisense RNA was used to the inhibition of SV40 DNA replication.

It is known that the ori-core region is the portion that the melting and untwisting of DNA is brought about by the binding of T Ag (Buchman and Gralla, 1990; Parsons *et al.*, 1991; Parsons and Tegtmeyer, 1992) when SV40 DNA replication is initiated. The formation of complex between SV40 DNA replication origin and T Ag is first change for the initiation of DNA replication. First, a monomer of T Ag binds to pentadecadeoxyribonucleotides 2 to nucleate the assembly of an early hexamer. Second, ATP stimulates the coorperative assembly of additional T Ag to form a hexamer on the early half of the origin through monomer-monomer interactions. Third, the early hexamer enhances the cooperative assembly of the weaker-binding late hexamer on DNA through hexamer-hexamer interactions. Fourth, the structural changes in hexamers melt imperfect inverted domain on early DNA and untwisting AT domain on late DNA. Fifth, the melting and untwisting release the hexamers from the pentanucleotides and allow them to act as a helicase to extend the origin replication bubble in opposite directions. At

this time, T Ag hexamer on early DNA is migrated to the early DNA by helicase activity and T Ag-DNA polymerase complex is formed and then RNA primer is formed by primase activity. This results in the formation of the first nascent chain.

At this time, on the supposition that antisense RNA is base-paired to the target site on the early DNA and then the synthesis of RNA primer by primase is affected, the experiment was done. In the treatment with antisense RNA I or BS, the replication was inhibited by 10%. In the treatment with antisense RNA II or SN, the replication was inhibited remarkedly: by II, 40% inhibition and SN, 80% inhibition. As late T Ag hexamer have a helicase activity by structural change, the complex is formed between T Ag and DNA pol α-primase and migrated to the direction of late DNA for the synthesis of a second nascent chain. Thus, it is assumed that when antisense RNA II or SN is bound to the target that was formed transiently, RNA primer synthesis by T Ag-DNA pol α-primase complex was inhibited. Because RNA primer synthesis for the synthesis of a second nascent chain occurs after the formation of RNA primer for the synthesis of the first nascent chain, it is possible that antisense RNA II or SN inhibits DNA replication by its inhibitory role when RNA primer is formed to synthesize a second nascent chain. In the treatment with antisense RNA I+SN, the replication was inhibited by 85%; in the situation in which the effect of I is slight, this high level of the inhibition results from the strong inhibition by SN. In the treatment with antisense RNA BS+SN, the replication was inhibited by 65%, similar to the inhibitory rate by antisense RNA I+SN.

Unexpectedly, in the treatment with I+II and II+BS it was shown that the replicative rate is enhanced by 10% and 20%, respectively. Because the inhibitory aspect of gene expression that was reported from the investigations by antisense tool known up to date was an inhibitory effect, the phenomenon that SV40 DNA replication was enhanced offers a paradox. However, it is known that the intracellular degradation of antisense molecules has important implications. Several studies have demonstrated that nucleotides and nucleosides can themselves increase cell proliferation (Huang *et al.*, 1989; Wang *et al.*, 1990) as well as cell migration (Kartha and Toback, 1992). Thus, as antisense RNA is degraded, the breakdown products may exert mitogenic effect that competes with the intact molecule's antiproliferative effects.

In the treatment with antisense RNA II and/or BS rather than I and/or SN, it was shown that the inhibitory rate of RNA primer synthesis differed from that of DNA replication: the inhibitory level of DNA replication is larger than that of RNA primer synthesis. Thus, it is suggested that in addition to the binding on primase initiation sites as a target, antisense RNA II and/or BS have a nonspecific effect: indirect inhibition of antisense RNA by the binding to the enzyme(s) and/or factor(s) which are related to DNA replication. Actually, in the binding assay of antisense RNA and COS nuclear extract, it was shown that BS has affinities to proteins (data not shown). And it is reported that the binding of antisense molecules to proteins can alter the protein's biological function (Bock *et al.*, 1992). Thus, it is possible that the binding of antisense RNA to the proteins, especially replication-related proteins, can inhibit DNA replication indirectly.

In conclusion, this work can be summarized into the following three points. First, it shows that DNA replication as function controlled was inhibited by the binding of antisense RNA. Second, this inhibitory effect of DNA replication was proven by measurement of RNA primer formation as a control level. Third, in some cases the enhancement of the inhibitory effect of antisense RNA on DNA replication rather than primer formation reflects the indirect effect by the binding of antisense RNA to replication-related proteins.

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