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Initiation of Simian Virus 40 DNA Replication In Vitro

HIROYOSHI ARIGA†* AND SUMIO SUGANO

Department of Virology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan

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Exogenously added simian virus 40 (SV40) DNA can be replicated semiconservatively in vitro by a mixture of a soluble extract of HeLa cell nuclei and the cytoplasm from SV40-infected CosI cells. When cloned DNA was used as a template, the clone containing the SV40 origin of DNA replication was active, but a clone lacking the SV40 origin was inactive. The major products of the in vitro reaction were form I and form II SV40 DNAs and a small amount of form III. DNA synthesis in extracts began at or near the in vivo origin of SV40 DNA synthesis and proceeded bidirectionally. The reaction was inhibited by the addition of anti-large T hamster serum, aphidicolin, or RNase but not by ddNTP. Furthermore, this system was partially reconstituted between HeLa nuclear extract and the semipurified SV40 T antigen instead of the CosI cytoplasm. It is clear from these two systems that the proteins containing SV40 T antigen change the nonspecific repair reaction performed by HeLa nuclear extract alone to the specific semiconservative DNA replication reaction. These results show that these in vitro systems closely resemble SV40 DNA replication in vivo and provide an assay that should be useful for the purification and subsequent characterization of viral and cellular proteins involved in DNA replication.

The genome of simian virus 40 (SV40) represents a simple model for a single mammalian cell replicon. SV40 DNA is a circular duplex molecule of about 5.2 kilobases (kb). The complete nucleotide sequence of SV40 is known (9, 29), and the structure and genetic organization have been studied extensively (3, 7, 23, 39).

SV40 DNA replication begins at a unique site and proceeds bidirectionally (4, 8). Although the initiation of SV40 DNA replication in vitro has never been conclusively demonstrated, considerable insight into this process has been gained by genetic analysis of the viral replication functions and by studies with purified T antigen (35, 37). The site of initiation for DNA synthesis has been located on the viral genome by electron microscopic analysis of replicative intermediates (8) and by analysis of the distribution of radioactivity in pulse-labeled viral DNA (4). Recently, the exact nucleotides at which replication begins were also reported (14).

T antigen and the structurally related D2 hybrid protein produced by the adenovirus SV40 hybrid Ad2^{+}D2, bind specifically to SV40 DNA or SV40 chromatin at or near the origin of replication (13, 20, 28, 31, 37, 38). Tjian has shown that the D2 protein binds sequentially to three tandemly arranged binding regions in the origin region (38). It seems likely that the interaction between T antigen and binding site II, which is located in a 27-base pair perfect palindrome (9, 29), is necessary for the initiation of viral DNA replication (25, 26, 32).

The replication of SV40 DNA appears to be semidiscontinuous. The leading strand grows by a continuous process, and the lagging strand, primed with RNA polymerase, is synthesized discontinuously (14, 17, 27). There is little direct evidence to indicate which, if any, of the known cellular DNA polymerases carries out SV40 DNA synthesis. Several indirect lines of evidence suggest that DNA polymerase α is the predominant replicative enzyme (24, 40).

In the present paper, a soluble enzyme system capable of replicating exogenously added SV40 DNA is described. DNA synthesis carried out in this in vitro system closely resembles SV40 DNA replication in vivo.

MATERIALS AND METHODS

Cells and virus. The SV40-transformed monkey cell CosI (10, 11) was obtained from Y. Gluzman. SV40 was propagated in the monkey cell line GC7, and SV40 DNA was extracted from purified virions by CsCl-ethidium bromide equilibrium centrifugation as described previously (41).

Preparation of nuclear extract and cytoplasm. All of the following procedures have been described previ-

† Present address: Department of Microbiology, School of Medicine, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, NY 11794.

ously in detail (2, 16, 21). HeLa nuclei in hypotonic buffer were prepared by Dounce homogenization of cells which were grown in suspension culture. The nuclei, which were rapidly frozen in liquid nitrogen, were thawed and extracted with 100 mM NaCl at 0°C for 5 min. The HeLa nuclear extract was freed of insoluble material by centrifugation at 20,000 × *g* for 20 min. Cytoplasm was prepared from the monolayer cultures of CosI cells which had been infected for 40 h with SV40 at a multiplicity of infection of 100 PFU per cell. Crude CosI cell cytoplasm was prepared by Dounce homogenization as previously described for adenovirus-infected HeLa cells (16). After centrifugation of crude cytoplasm at 100,000 × *g* for 30 min, the supernatant was precipitated with 60% $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ precipitate was dissolved in a solution of 25 mM Tris (pH 7.5), 7 mM β-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, and 50 mM NaCl, dialyzed against the same buffer, and used as cytoplasm in the following experiments.

Conditions for in vitro reaction. Reaction mixtures (100 μl) contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.05 mM each dATP, dGTP, and dTTP, 1.5 μM [α-^{32}P]dCTP (410 Ci/mol, 5,000 to 15,000 cpm/pmol), 3.75 mM ATP, 20 μl of HeLa nuclear extract (protein concentration, 2.5 mg/ml), 5 μl of CosI cytoplasm (protein concentration, 10 mg/ml) or 0.3 μg of purified proteins containing T antigen (the amount of T antigen was approximately 0.12 μg), and 0.2 μg of form I of SV40 or the clone containing origins of SV40 DNA synthesis (see below). After incubation of the reaction mixture for 60 min at 37°C and subsequent digestion with pronase (10 μg) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol with or without phenol extraction. The DNA was dissolved in 50 μl of 40 mM Tris–1 mM EDTA–5 mM sodium acetate (pH 7.8) and electrophoresed on 1% agarose gels in the same buffer. The gels were dried and autoradiographed on Kodak X-ray film XRS.

Construction of clones containing SV40 DNA. The early region of the SV40 genome (*Eco*RI-*Pvu*II site spanning 0 to 0.71 map unit) was inserted into *Eco*RI-*Pvu*II sites of plasmid pBR322 and propagated in *Escherichia coli* HB101 (22). This cloned DNA was designated pMTI. A plasmid defective in the SV40 origin (*ori*) region (pMTIOD) was constructed from pMTI by deleting the unique SV40 *Bgl*I site at 0.66 map unit as follows. An *ori*-defective plasmid was prepared by partial digestion of pMTI with *Bgl*I and nuclease S1, followed by religation of the DNA by the procedure described by Gluzman (11). Clones missing from the SV40 *Bgl*I site were screened and propagated. pMTIgpt and pMTIgptOD were constructed from pMTI and pMTIOD by an addition of the *E. coli* gene (xanthine-guanine phosphoribosyl-transferase) at the *Eco*RI site. pSVO and pSVOD containing the origin and early promoter regions of the SV40 genome (*Hin*dIII-*Pvu*II site spanning 0.64 to 0.71 map unit) were constructed from pMTI and pMTIOD, respectively. After pMTI or pMTIOD was digested with *Hin*dIII and *Pvu*II, the *Hin*dIII-*Pvu*II origin fragment of the SV40 genome was isolated and inserted into the same sites of pBR322. Plasmids pMTIOD, pMTIgptOD, and pSVOD had deletions of six nucleotides at the *Bgl*I site of SV40 DNA (0.66 map unit).

Construction of the Ad-SV hybrid virus and purification of the SV40 T antigen. To purify the SV40 T antigen efficiently, we constructed the adenovirus-SV40 recombinant (designated Ad-SV) as originally reported by Solnick (33) with some modification. A fragment of SV40 DNA containing the entire early transcriptional unit (*Hin*dIII-*Bam*HI site spanning 0.64 to 0.14 map unit), apart from the promoter region, was joined to a small fragment of the adenovirus genome containing the major late promoter spanning from 15.5 to 16.6 map units. In this way, the relatively weak SV40 early promoter was replaced by the strong adenovirus late promoter, but the coding sequence was left unchanged. After cloning in the plasmid pBR322, the hybrid fragment, consisting of the adenovirus major late promoter and the SV40 early gene, was inserted into the adenovirus genome by substitution for the sequence between 75.9 and 85.5 map units, such that the inserted promoter is oriented in the same direction as the resident late promoter at 16.6 map units. After the sequential screening by plaque purification on human 293 cells and two passages in the GC7 line of African green monkey kidney cells (41), we isolated Ad-SV. This recombinant can grow well in both HeLa and CV1 cells. A virus stock of Ad-SV was grown on CV1 or GC7 cells for use in the following experiments. Ad-SV DNA, purified from the Ad-SV virion, was exactly the same as that determined by restriction enzyme analysis. Anti-T sera precipitate authentically sized T antigen after infection in HeLa cells with the hybrid virus. Therefore, this T antigen produced from Ad-SV is not a hybrid protein like the D2 protein from Ad$^+$D2. The relative amount of T antigen produced in HeLa cells infected with Ad-SV was approximately three times that in SV40-infected CV1 cells (data not shown). The purification of T antigen from Ad-SV-infected cells was carried out by the procedure reported by Tegtmeyer and Anderson (36). Both radioimmunoassay (30) and ATPase assay (1) were used as a purification monitor through all the steps (see Table 1). The purity was approximately 50% in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The main contaminant was a 100,000-molecular-weight protein which was a late gene product of the adenovirus. It seems unlikely that this late protein has any effect on viral DNA replication (3). Therefore, we used this partially purified T antigen in the following experiments.

Materials. Anti-T hamster serum prepared from tumor-bearing animals was kindly provided by N. Yamaguchi. All of the restriction enzymes were purchased from Bethesda Research Laboratories. [α-^{32}P]pCTP was from Amersham Corp.

RESULTS

DNA synthesis on exogenous templates by soluble extracts of HeLa nuclei and CosI cytoplasm. The in vitro replication activity of mixtures of uninfected HeLa nuclear and SV40-superinfected CosI cytoplasmic extracts was measured on form I of the two cloned DNAs as a template (Fig. 1). These two clones (pMTIgpt and pMTIgptOD) consist of the *Eco*RI-*Pvu*II fragment of SV40 (0 to 0.71 map unit), the large *Eco*RI-*Pvu*II fragment of pBR322 DNA, and the

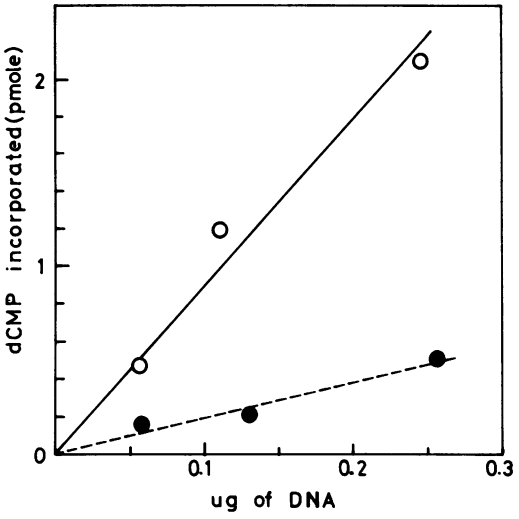

FIG. 1. DNA synthesis in vitro on cloned SV40 DNA sequences. The reaction was carried out with increasing amounts of pMTIgpt or pMTIgptOD as a template as described in the text. After 60 min at 37°C, the acid-insoluble radioactivity was counted. Symbols: (○) pMTIgpt, (●) pMTIgptOD.

E. coli gpt gene. The 0 to 0.71 portion of SV40 in these clones includes both the origin for DNA replication and the early region (9, 29), which contains three T antigen binding sites (38). The only difference between the two clones is that pMTIgptOD is missing the *BglI* site of SV40 DNA, which is located in T antigen binding site II (9, 11, 29). It was determined that pMTIgptOD, but not pMTIgpt, lost the origin activity after the replicative activity was measured by transfection of DNA into CosI cells expressing authentic SV40 T antigen (10) (data not shown).

When the template activities of pMTIgpt and pMTIgptOD were compared, a clear difference of incorporation of radioactive deoxynucleosides was observed (Fig. 1). Although incorporation was linear with either DNA, 4.5 times the incorporation (9 versus 2 pmol/μg of DNA) was obtained, with pMTIgpt DNA containing the *BglI* site of SV40. The same results were also obtained when pSVO and pSVOD, derivatives of pMTIgpt and pMTIgptOD, respectively, were used as templates (Table 1). This result suggests that in the soluble extract system, T antigen binding site II was necessary, therefore reflecting requirements for an in vivo DNA synthesis.

Requirements for SV40 DNA synthesis. DNA synthesis was performed within various components of the reaction mixture, and the products were visualized by autoradiography after neutral agarose gel electrophoresis (Fig. 2). The complete reaction containing both HeLa nuclear extract and SV40-infected CosI cytoplasm on SV40 DNA produced forms I, II, and III, which were about 30, 50, and 20%, respectively. Higher-molecular-weight DNAs, possibly concatemers, were also observed. The reaction without the SV40 DNA template yielded absolutely no bands. These results showed that the reaction was dependent on the exogenously added DNA and that the cytoplasm from SV40-infected CosI cells contained no functional SV40 DNA. The reaction with HeLa nuclear extract alone yielded a small amount of form I and large amounts of forms II and III. These latter DNAs were mainly derived from a repair-type reaction as described

TABLE 1. Reaction specificity[a]

Reaction	cpm incorporated (pmol)		pSVO/pSVOD ratio
	pSVO	pSVOD	
HeLa NE + Cos Cyt	26,000 (2.60)	3,310 (0.33)	7.86
HeLa NE	35,600 (3.56)	28,600 (2.87)	1.24
HeLa NE + T Ag	38,900 (3.89)	4,780 (0.48)	8.14
Cos Cyt	4,570 (0.46)	1,150 (0.12)	3.97
T Ag	480 (0.05)	162 (0.02)	2.96

[a] SV40 T antigen (T Ag) was purified from 12 liters of the suspension cultures of HeLa cells (3.0×10^5 cells per ml) which had been infected for 48 h with Ad-SV at a multiplicity of infection of 50 PFU per cell. The purification procedure reported by Tegtmeyer and Andersen (36) was used; purification steps included nuclear extraction in buffer containing 10 mM HEPES (pH 8.0) and 10 mM NaCl, ammonium sulfate precipitation, hydroxylapatite chromatography, hydrophobic interaction chromatography (phenyl-Sepharose 4B), and DEAE-Sephacel chromatography. The radioimmunoassay (30) and ATPase assay (1) were used to monitor purification. The final yields of total protein and T Ag were approximately 90 and 40 μg, respectively. The final volume of proteins containing T Ag was 150 μl (protein concentration, 0.6 mg/ml), and the protein recovery was 2%. The in vitro reaction was carried out as described in the text. In this reaction, 20 μl of HeLa nuclear extract (NE) (protein concentration, 2.5 mg/ml), 5 μl of CosI cytoplasm (Cos Cyt) (protein concentration, 10 mg/ml), or 0.3 μg of purified proteins containing T Ag (approximately 0.12 μg of T antigen), and 0.4 μg of form I pSVO or pSVOD DNA were used. After incubation of the reaction mixture for 60 min at 37°C, the acid-insoluble radioactivity incorporated was counted.

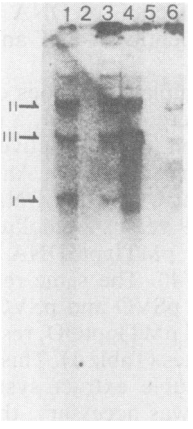

FIG. 2. Requirements for SV40 DNA synthesis in vitro. The synthesis was carried out on 0.2 μg of SV40 DNA as a template, using various reaction conditions. After 60 min at 37°C, the reaction mixture was treated with pronase and sodium dodecyl sulfate, and the DNA was precipitated with ethanol. DNA was visualized by autoradiography after agarose gel electrophoresis. Lane 1, complete reaction containing SV40 DNA, SV40-infected CosI cytoplasm, uninfected HeLa cell nuclear extract, $MgCl_2$, and ATP; lane 2, minus SV40 DNA; lane 3, minus the cytoplasm from SV40-infected CosI cells; lane 4, minus the nuclear extract from HeLa cells; lane 5, minus $MgCl_2$; lane 6, minus ATP. The total incorporated dCMP amounts estimated by counting 0.1 ml of the mixture were 17.5, 0.05, 14.0, 12.5, 0.25, and 4.0 pmol/μg of added DNA in lanes 1 to 6, respectively. The horizontal bars represent the positions of SV40 form I, II, and III DNAs.

below. Similarly, the DNAs produced by CosI cytoplasm alone were the result of repair-type reactions. We have to stress that, although the reactions either by HeLa nuclear extract or by CosI cytoplasm alone seem to be similar to that by complete reaction in terms of acid-precipitable counts and patterns on neutral agarose gels, the products of the reactions are quite different (see below). The synthesis reaction directed by both HeLa nuclear extract and CosI cytoplasm was absolutely dependent on Mg^{2+} and ATP. The product produced without added ATP was probably derived from a repair-type reaction or by utilization of ATP originally involved in crude extracts.

Since SV40 DNA replication absolutely depends on SV40 T antigen, anti-T serum from a tumor-bearing hamster was added to the reaction mixture containing both HeLa nuclear extract and CosI cytoplasm (Fig. 3). The anti-T serum used here immunoprecipitated large T antigen but not small t antigen from extracts of infected CV1 cells (data not shown). Furthermore, this anti-T serum did not inhibit any DNA polymerases purified from HeLa cells. Anti-T serum reduced the incorporation to 40% of the control, whereas normal serum from unimmunized hamster serum allowed 90% of the synthesis of control reactions. The incorporation of radiolabel after the addition of anti-T serum consisted of reduced amounts of forms II and III and no form I, and there was little change after exposure to normal serum. These results strongly suggest that this in vitro system is dependent on T antigen. Addition of aphidicolin, which is a strong inhibitor of DNA polymerase α (19), or the addition of RNase also inhibited the reaction by 60–70%, respectively. *N*-Ethylmaleimide, which is an inhibitor of both DNA polymerases α and β, also inhibited the reaction (data not shown), and ddTTP, which is an inhibitor of both polymerases β and γ, had no effect even after the ratio of ddTTP to dTTP was increased to three. α-Amanitin, which is an inhibitor of RNA polymerases I and II, had no effect (data not shown). These results were similar to those for the in vivo situation, in which DNA polymerase α may elongate RNA primers (24, 40). When pMTIgpt was used as a template, the same results shown in Fig. 2 and Fig. 3 were obtained (data not shown).

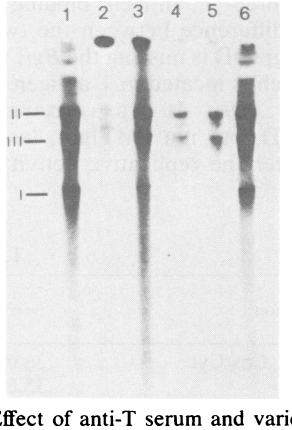

FIG. 3. Effect of anti-T serum and various inhibitors on the DNA polymerase reaction. All reactions were carried out on 0.2 μg of SV40 DNA. When serum (10 μl) was added to the reaction, the mixture was held for 10 min at 4°C before the synthesis was started at 37°C. After 60 min at 37°C, the DNA from all of the reaction mixtures was processed as for Fig. 2. The total (picomoles per microgram of added DNA) and percent incorporation as compared with control experiments are also shown below. Lane 1, control reaction, 14.60 pmol, 100%; lane 2, plus anti-T hamster serum, 5.85 pmol, 40%; lane 3, plus normal hamster serum, 13.90 pmol, 95%; lane 4, plus 50 μg of RNase A per ml; 4.85 pmol, 33%; lane 5, plus 10 μg of aphidicolin per ml, 5.90 pmol, 40%; lane 6, plus 30 μM of ddTTP, 14.50 pmol, 99%.

Size distribution of SV40 DNA synthesized in vitro. SV40 DNA synthesized in vitro was analyzed by sedimentation through alkaline sucrose gradients. The coupled reaction between HeLa nuclear extract and SV40-CosI cytoplasm gave the following distribution. Denatured supercoiled, single-stranded circular, and single-stranded linear DNAs were 23, 41, and 36%, respectively, whereas the reaction by HeLa nuclear extract alone showed 1, 31, and 37%, respectively (Fig. 4). Thirty-one percent of the molecules were smaller than the single-stranded linear molecules (16S). The reaction by cytoplasm alone from SV40-infected CosI cells was similar to the pattern of HeLa nuclear extract alone.

We emphasize again that DNA products, especially single-stranded circular and single-stranded linear DNAs, have been made by different mechanisms in the complete and partial reaction mixtures. When the reactions were carried out for only 10 min at 37°C (pulse experiment) and the products were analyzed by sedimentation through neutral sucrose gradients, the putative intermediate (25S) appeared only in the coupled reaction, but neither in HeLa nuclear extract alone nor in CosI cytoplasm alone. Furthermore, the single-stranded linear DNA and the small DNA (4S, Okazaki fragment) were seen after the intermediate forms isolated from the neutral sucrose gradient were applied to alkaline sucrose gradients (data not shown). Therefore, it could be concluded that the DNA molecules produced in the reaction mixture containing both HeLa nuclear extract and CosI cytoplasm are derived from replication intermediates. On the other hand, DNA molecules with a broad size range apparently arise from random nicking and ligation of the template DNA (repair synthesis) in the reactions containing HeLa nuclear extract or CosI cytoplasm alone.

Evidence for semiconservative replication. To rule out the possibility that the in vitro product consists of short DNA chains that are covalently linked to the template DNA, SV40 DNA was synthesized in the reaction mixture containing dBrUTP in place of dTTP and analyzed by neutral CsCl equilibrium centrifugation (Fig. 5). About 8% of the in vitro product was fully substituted with bromouracil, 81% banded at hybrid density, and the densities were slightly greater than that of the unsubstituted marker SV40 DNA. When the reaction was carried out only by HeLa nuclear extract, as expected from the preceding experiments, a broad distribution pattern was obtained at densities between those of half- and unsubstituted molecules. The reaction by SV40-infected CosI cytoplasm alone showed that only 10% of the molecules were partially substituted and 90% were unsubstituted (data not shown). These data are consistent with the hypothesis that the mixed extracts can initiate SV40 DNA synthesis de novo and that the CosI cytoplasm containing SV40 T antigen can change the reaction of HeLa nuclear extract from the repair to semiconservative replication.

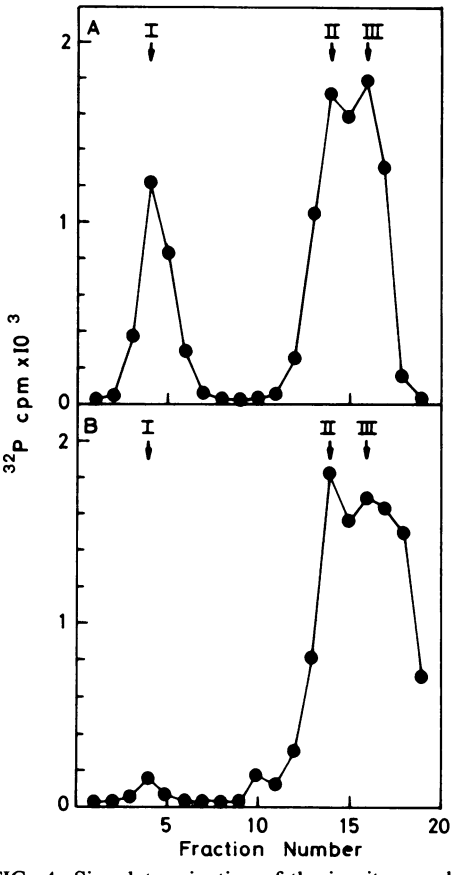

FIG. 4. Size determination of the in vitro product by alkaline sucrose gradient. After the reaction on SV40 DNA for 60 min at 37°C, DNA was extracted with phenol saturated with 10 mM Tris-hydrochloride (pH 8.1)–1 mM EDTA, and the sample was mixed with SV40 [^{3}H]DNA prepared by the Hirt method (15) from SV40-infected CV1 cells. The samples were loaded onto a 5 to 20% alkaline sucrose gradient containing 0.2 M NaOH, 0.5 M NaCl, and 10 mM EDTA and centrifuged in the SW50.1 rotor at 45,000 rpm for 2.5 h at 4°C. The acid-insoluble radioactivity was counted after fractionation. The arrows (I, II, and III) represent the positions of the denatured supercoiled, single-stranded circular, and single-stranded linear DNAs of SV40. The linear DNA is partly unit length and partly less than unit length. (A) Reaction with both HeLa nuclear extract and SV40-CosI cytoplasm. (B) Reaction with HeLa nuclear extract alone. The total incorporated dCMP amounts estimated by counting 0.1 ml of the reaction were 17.5 and 14.0 pmol/μg of added DNA in A and B, respectively.

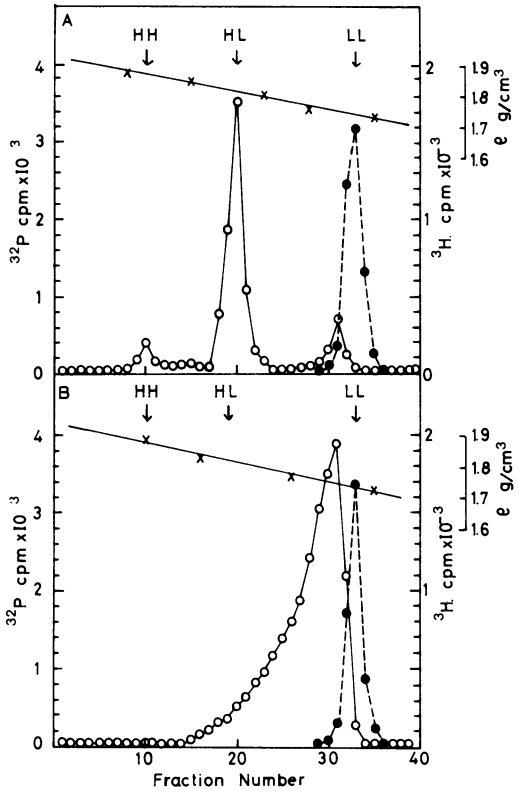

FIG. 5. Isopycnic centrifugation at a neutral pH of DNA synthesized in vitro in the presence of dBrUTP. A standard reaction mixture containing 30 μM each dATP, dGTP, and dBrUTP (in place of dTTP) and 1.5 μM [^{32}P]dCTP was carried out on SV40 DNA for 60 min at 37°C. The DNA was extracted with phenol after the treatment of the mixture with proteinase K (500 μg/ml) for 30 min at 37°C, precipitated with ethanol, dissolved, mixed with SV40 [^{3}H]DNA, and digested with *Eco*RI for 1.5 h at 37°C to be linearized. The density of the DNA sample containing 25 mM Tris (pH 7.4), 1 mM EDTA, and 0.1 M NaCl was adjusted to 1.7 g/cm^3 by the addition of CsCl and centrifuged in a VTi65 rotor at 48,000 rpm for 20 h at 25°C. After fractionation, the acid-insoluble radioactivity was counted, and the density was measured. The arrows indicate the expected positions for fully substituted (HH), hybrid (HL), and unsubstituted (LL) SV40 DNAs. (A) Reaction with both HeLa nuclear extract and SV40 CosI cytoplasm. (B) Reaction with HeLa nuclear extract alone. Symbols: (○) ^{32}P-labeled in vitro product; (●) SV40 [^{3}H]DNA. The total incorporated dCMP amounts estimated by the counting 0.1 ml of the reaction were 16.0 and 14.0 pmol/μg of added DNA in A and B, respectively.

Bidirectional DNA replication from the origin. One critical aspect of SV40 DNA replication is that replication starts at a unique origin and moves bidirectionally (4, 8). To assess this point, the DNA products synthesized for 5, 10, and 40 min were extracted and mixed with ^{3}H-labeled SV40 DNA prepared from the Hirt supernatant of SV40-infected CV1 cells (15), and the distribution of labeled DNA fragments was examined after digestion of DNA with *Bst*NI (Fig. 6). The ^{32}P/^{3}H ratio of radioactivity was normalized to that obtained in the *Bst*NI A fragment. In the first 5 min of the reaction, a symmetrical pattern of radioincorporation indicated that synthesis initiated at the center of the G fragment, in which the replication origin in

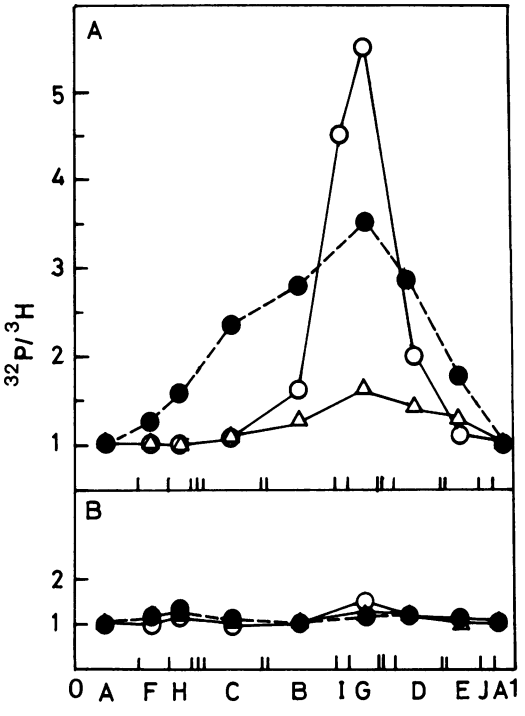

FIG. 6. Kinetics of labeling of various regions of SV40 DNA. After incubation for 5, 10, and 40 min, DNA was extracted with phenol after the treatment of the reaction with proteinase K (200 μg/ml) for 30 min at 37°C, mixed with SV40 [^{3}H]DNA, digested with *Bst*NI for 1.5 h at 37°C, and electrophoresed on a 2% agarose gel containing 40 mM Tris-hydrochloride (pH 7.8), 1 mM EDTA, and 5 mM sodium acetate. DNA bands were cut out after ethidium bromide staining, and radioactivity was counted. The ratio of [^{32}P]DNA (pulse-labeled) to [^{3}H]DNA (uniformly labeled) is expressed as a function of the position of each fragment on the genome. The ^{32}P/^{3}H ratio of the A fragment was arbitrarily set as 1, and the other values were normalized to it. The actual counts per minute in the A fragments were (^{32}P/^{3}H): (A) 85/2,000 at 5 min, 250/2,020 at 10 min, and 3,020/2,010 at 40 min; (B) 320/1,990 at 5 min, 530/2,050 at 10 min, and 2,970/2,010 at 40 min. (A) Reaction with both HeLa nuclear extract and SV40 CosI cytoplasm; (B) Reaction with HeLa nuclear extract alone. Symbols: (○) 5-min reaction; (●) 10-min reaction; (△) 40-min reaction.

vivo is known to be located (14). After 40 min, there was little variation in the labeling of any of the restriction fragments, which indicates that nearly the whole strand was replicated. In contrast, in the reaction by HeLa nuclear extract alone, all of the values were near one, as expected, even when the reaction was continued only for 5 min, which showed that the repair-type reaction occurs at random around the genome. When the reaction with SV40 CosI cytoplasm alone was carried out, the small peak of the G fragment appeared above a high background of the other fragments (data not shown), which reflects the previous finding that 10% of the reaction is semiconservative and the rest is repair-type. Furthermore, when the coupled reaction was performed on pMTIgpt or pMTIgptOD as a template, the same results were obtained. Although these experiments clearly showed that replication performed by HeLa nuclear extract and CosI cytoplasm was initiated at the origin-containing fragment and moved bidirectionally, the precise nucleotide at the origin cannot be located by this experiment.

Partial reconstitution of in vitro SV40 DNA replication system by HeLa nuclear extract and SV40 T antigen. To extend and analyze this system, we tried to reconstitute the system by using the purified T antigen. For the efficient purification of T antigen, we constructed the Ad-SV recombinant as originally reported to Solnick (33). The entire SV40 early transcriptional unit (*Hin*dIII-*Bam*HI site spanning 0.64 to 0.14 map unit), which was joined to the adenovirus 2 major late promoter spanning from 15.5 to 16.6 map units, was inserted into the adenovirus genome by substitution for the sequence between 75.9 and 85.5 map units. The constructed recombinant, Ad-SV, can grow well in both HeLa and CV1 cells. The T antigen produced from Ad-SV-infected HeLa cells had the same size as that of the original T antigen from SV40-infected CV1 cells. The in vitro replication activity of the mixture was measured on two cloned DNAs as a template (Table 1). These two clones (pSVO and pSVOD), which are derivatives of pMTIgpt and pMTIgptOD, respectively, consist of a *Hin*dIII-*Pvu*II fragment of SV40 (0.64 to 0.71 map unit) and the large *Hin*dIII-*Pvu*II fragment of pBR322. The 0.64-to-0.71 map unit portion of SV40 in these clones includes the origin for DNA replication (39), which contains three T antigen binding sites (37). The only difference between the two clones, as for pMTIgpt and pMTIgptOD, is that pSVOD is missing the *Bgl*I site of SV40 DNA, which is located in T antigen binding site II (37). It was determined that pSVOD, but not pSVO, lost replication activity in CosI cells expressing authentic SV40 T antigen (11) (data not shown). When the template activity of two clones was compared in the mixture of different combinations, the original combination mixture (HeLa nuclear extract and SV40-infected CosI cytoplasm) gave rise to a high ratio of incorporation (pSVO/pSVOD), 7.86, as shown in Fig. 1. Although significant incorporation was obtained in the reaction of HeLa nuclear extract alone on the two clones, analysis showed that HeLa nuclear extract alone has no or little specificity for T antigen binding site II. After the addition of T antigen to the HeLa nuclear extract, the specificity was restored (the ratio was 1.24 to 8.14). When the reaction was carried out only by SV40-infected CosI cytoplasm or T antigen alone, the radioactivity incorporated was quite small, but the specificity was still evident to a small extent. These results clearly show that purified T antigen can substitute for the SV40-infected CosI cytoplasm; both show a dependence on T antigen binding site II, which is the specific DNA replication site of SV40.

To determine the initiation point of replication in the reconstituted system, the DNA products synthesized after 5 and 60 min of reaction were extracted and digested with *Hin*dIII and *Pvu*II, and the distribution of labeled DNA fragments was examined (Table 2). The digestion of DNA with two enzymes yielded two fragments of 2,328 and 342 base pairs. The small fragment contains the origin of SV40 DNA replication. The ratio of the radioactivity of the small fragment to the large fragment was normalized to the ratio of the molecular weights of the two fragments. After 60 min, there was little variation in the ratio of radioactivity obtained by all three combinations, which indicates that nearly the whole strand was replicated. In the first 5 min of the reaction performed with both SV40-infected CosI cytoplasm and T antigen combined with HeLa nuclear extract, a very high ratio was obtained. In contrast, in the reaction with HeLa nuclear extract alone, the value was still near one. These results clearly indicate that DNA synthesis starts at the origin-containing fragment in the reaction including the SV40-infected CosI cytoplasm or SV40 T antigen.

To further examine the involvement of T antigen in the reaction on pSVO as a template, anti-T serum from a tumor-bearing hamster was added to the mixture, and the products were visualized by autoradiography after neutral agarose gel electrophoresis (Fig. 7). In the course of the experiments shown in Fig. 1 to 6, we found that the patterns on the neutral agarose gels were not always parallel to that of the acid-insoluble radioactivity incorporated in the reaction mixture, especially when the anti-T serum and several inhibitors were added. When T antigen was added to the reaction mixture, for

TABLE 2. Identification of the initiation point of the reaction[a]

Reaction[b]	Time (min)	cpm incorporated		Small fragment/ large fragment ratio	Normalized ratio
		Small fragment	Large fragment		
HeLa NE + Cos Cyt	5	264	117	2.25	15.30
	60	1,130	7,260	0.15	1.06
HeLa NE	5	225	1,120	0.20	1.34
	60	2,260	11,350	0.20	1.36
HeLa NE + T Ag	5	543	321	1.69	11.50
	60	2,260	13,400	0.17	1.15

[a] The in vitro reaction was carried out on 0.4 μg of pSVO in various combinations as in Table 1. After incubation of the reaction mixture for 5 or 60 min at 37°C and subsequent digestion with pronase (10 μg) in sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was extracted with phenol saturated with 10 mM Tris-hydrochloride (pH 8.1) and 1 mM EDTA and precipitated with ethanol. The DNA was dissolved in water, mixed with 1.5 μg of cold pSVO, digested with *Hin*dIII and *Pvu*II, and electrophoresed on 1.7% agarose gel containing 40 mM Tris-hydrochloride (pH 7.8), 1 mM EDTA, 5 mM sodium acetate, and 0.5 μg of ethidium bromide per ml. The DNA bands were cut out after illumination under UV light, and the radioactivity was counted. The ratio of the incorporation of small fragment to large fragment was normalized to the ratio of the nucleotide numbers of the two fragments. The nucleotide numbers of large and small fragments are 2,328 and 342 base pairs, respectively (ratio, 6.8).

[b] NE, Nuclear extract; Cos Cyt, SV40-infected CosI cytoplasm; T Ag, protein fraction containing T antigen.

example, almost all of the radioactivity was found at the origin of the gel, and little was located at the positions of form I, II, and III DNAs, although the acid-insoluble counts incorporated were still 40% of the control without the addition of anti-T serum (Fig. 3). These DNA samples had been loaded on the agarose gels after the reaction mixtures were treated with sodium dodecyl sulfate and proteinase K followed by ethanol precipitation without phenol extraction (Fig. 2 and 3).

This inconsistency between the gel patterns and the acid-insoluble radioactivity incorporated was resolved by counting the radioactivity after phenol extraction of DNA in the reaction mixture. Therefore, the supposed complex of T antigen-DNA-anti-T serum seen at the top of the gel in Fig. 3 could not be seen in Fig. 7 due to the partition of this complex into the interphase after phenol extraction of the reaction mixture. Quite similar patterns were obtained in the reaction by SV40-infected CosI cytoplasm and T antigen combined with HeLa nuclear extract (lanes 1 to 3 and 7 to 9). The products were forms I, II, and III, and other forms consisted of closed circular DNA with different numbers of superhelical forms because *Eco*RI converted all forms to linear DNA form III. The form I product showed a broad distribution band in this experiment, whereas the form I products shown in Fig. 2 and 3 were relatively homogeneous. These variations were sometimes observed in different experiments. When the cloned DNA containing the SV40 origin was used, these broad form I DNAs were often observed. We do not know the reason for this phenomenon at present. The pattern of incorporation after the addition of anti-T serum showed no detectable bands (lanes 2 and 8), whereas there was little reduction by normal serum (lanes 3 and 9). In contrast, in the reaction by HeLa nuclear extract alone, there was little change of patterns of the products after the addition of anti-T serum (lanes 4 to 6). As shown in Fig. 2, 3 and 7, some differences were observed. However, we have to mention that the reaction by HeLa nuclear extract alone, even when the form I product could be seen on the neutral agarose gels, was either the repair-type reaction shown in Fig. 5 or the nonspecific DNA replication reaction in which initiation occurred at random through the strands.

These results clearly showed that semipurified T antigen can be completely substituted for the cytoplasm of SV40-infected CosI cells, and that T antigen can change the reaction performed by HeLa nuclear extract from the repair-type to the semiconservative-type and from a nonspecific to a specific reaction.

DISCUSSION

In this communication, we have described the development of a system which replicates SV40 DNA in soluble extracts of HeLa cell nuclei and the cytoplasm of CosI cells infected with SV40. Furthermore, this system was partially reconstituted with purified SV40 T antigen instead of CosI cytoplasm. This system carried out the semiconservative DNA replication of exogenously added SV40 DNA in vitro. The ability of

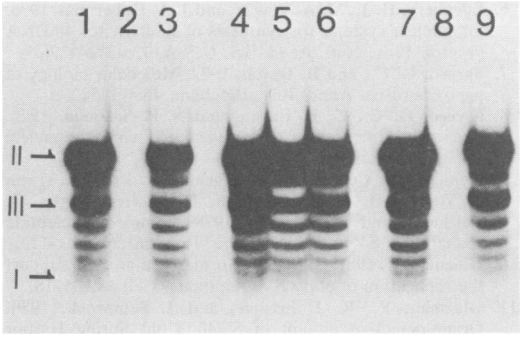

FIG. 7. Effect of anti-T serum on the reaction. The in vitro reaction was carried out on 0.4 μg of pSVO. When serum (10 μl) was added to the reaction, the mixture was held for 10 min at 4°C before the synthesis was started at 37°C. After 60 min at 37°C, the DNA was extracted as for Table 2 and electrophoresed on a 1.2% agarose gel containing the same buffer as that for Table 2. The DNA bands were visualized after the gels were dried and autoradiographed on Kodak X-ray film OH-I. Lanes 1 to 3, reaction with HeLa nuclear extract and SV40-infected CosI cytoplasm; lanes 4 to 6, reaction with HeLa nuclear extract alone; lanes 7 to 9, reaction with HeLa nuclear extract and purified T antigen. Lanes 1, 4, and 7, addition of no serum; lanes 2, 5, and 8, addition of anti-T hamster serum; lanes 3, 6, and 9, addition of normal hamster serum. The total incorporated dCMP amounts estimated by the counting of 0.1 ml of the mixture after phenol extraction were 6.75, 0.025, 6.25, 7.75, 7.25, 7.50, 7.25, 0.025, and 7.00 pmol/μg of added DNA in lanes 1 to 9, respectively.

this system to initiate replication on an exogenous template is in contrast to all previously reported subcellular DNA replication systems of SV40, which in vitro could only elongate the strands initiated in vivo (5, 6, 12, 34).

Several characteristics of the in vitro system described in this communication support its biological relevance. This system specifically requires the cytoplasm from SV40-infected CosI cells. The HeLa nuclear extract without addition of SV40-infected CosI cytoplasm yielded only a repair-type reaction. Although CosI cells are CV1 cells transformed with origin-defective SV40 DNA (10) and produce biologically active authentic T antigen, the cytoplasm from uninfected CosI cells did not support semiconservative replication of SV40 DNA (data not shown). The same situation was also observed when the cytoplasm from SV40-infected CV1 cells was added to the reaction mixture containing HeLa nuclear extract. It is known that less T antigen is produced in CV1 cells infected with SV40 than in similarly infected CosI cells (10, 11). This suggests that the amount of T antigen was not enough to promote DNA synthesis. This is strongly suggested by the partially reconstituted system, where semipurified T antigen is substituted for the CosI cytoplasm as shown above.

It was concluded that the reaction performed by the HeLa nuclear extract alone was dramatically changed from the nonspecific (repair-type) to the specific (replicative) reaction after the addition of either cytoplasm of CosI cells infected with SV40 or isolated T antigen. It is generally believed that T antigen is needed to initiate SV40 DNA replication by binding to specific sites located at or near the origin (37). This possibility was clearly supported by the comparison of two plasmid DNAs containing the early region of the SV40 genome with or without the *BglI* site. The plasmid DNA containing the *BglI* site located in T antigen binding site II had template activity in the in vitro system, whereas that missing the *BglI* site had little activity.

The product of the in vitro reaction was distributed in forms I, II, and III and concatemers. The reason for the presence of form III is not clear because form III is rarely detectable in vivo (3, 7, 23, 39). There seem to be three possibilities, one of which is that form III was derived from a repair-type reaction. The second possibility is that form I or form II was cleaved during or after the semiconservative synthesis. The third possibility is that form III is an intermediate, especially during segregation of the two daughter molecules, when both newly synthesized and template strands are cut transiently (3, 7, 23, 39).

The first possibility cannot be ruled out because about 10% of the products are probably derived from the repair-type reaction as evidenced by the density transfer experiment. However, all of the form III DNA, which was about 36% of the total product in the alkaline sucrose gradient, cannot be explained exclusively by the repair-type reaction. The second explanation also seems possible, because the exogenously added template of form I DNA was rapidly converted from form I to forms II and III in this crude extract after ethidium bromide staining, probably because of the presence of many endogenous nucleases. Indeed, the maximal extent of incorporation of precursors that we have observed corresponds to about 1% of the input DNA. However, we have to mention that only form I DNA could be a template in this system. When form II or form III DNA was used as a template in this system, only repair-type reactions occurred. It is likely that form III DNA is a real replicating intermediate. When the replicative intermediate isolated in a neutral sucrose gradient after a short incubation time of the mixture was loaded on an alkaline sucrose gradient, both full-sized linear single-stranded DNA and short DNA (putative Okazaki fragments) were observed. Furthermore, since form

I was indeed synthesized in this reaction, the segregation step must occur, and a transient form III must be present.

It seems likely that de novo initiation of DNA chains took place in this in vitro system. Replication forks began in the origin-containing fragment and moved bidirectionally. However, we cannot at present rule out the possibility that the input DNA included molecules that had initiated DNA synthesis in vivo. To clarify these two possibilities, we have to wait for the identification and localization of the first nucleotide incorporated, as has been done in vivo (14).

This reaction was inhibited by RNase and aphidicolin. These observations are quite consistent with many results obtained with SV40 DNA replication in vivo, suggesting the presence and function of RNA primers and DNA polymerase α. However, these results must be interpreted cautiously because they varied with changes in experimental conditions (18). Since RNA primers must be hybridized to a DNA strand, RNase A could not degrade them. It might be possible, however, that a conformational change susceptible to RNase A occurs at an origin of DNA replication in this crude system. The system described in this work should prove valuable for probing the biochemical mechanism of SV40 DNA replication. The requirement of the system for exogenous DNA offers an opportunity to define the specific structural features of the template, especially by using cloned DNA. In addition, the development of this system makes possible a rational approach to the purification and functional characterization of viral and cellular proteins involved in SV40 DNA replication.

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