MOLECULAR AND CELLULAR BIOLOGY, Aug. 1984, p. 1476–1482
0270-7306/84/081476-07$02.00/0

Vol. 4, No. 8

Replication of Cloned DNA Containing the *Alu* Family Sequence During Cell Extract-Promoting Simian Virus 40 DNA Synthesis

HIROYOSHI ARIGA

Department of Virology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokane-dai, Minato-ku, Tokyo 108, Japan

Received 18 January 1984/Accepted 25 April 1984

The replicating activity of several cloned DNAs containing putative origin sequences was examined in a cell-free extract that absolutely depends on simian virus 40 (SV40) T antigen promoting initiation of SV40 DNA replication in vitro. Of the three DNAs containing the human *Alu* family sequence (BLUR8), the origin of (*Saccharomyces cerevisiae* plasmid 2μm DNA (pJD29), and the yeast autonomous replicating sequence (YRp7), only BLUR8 was active as a template. Replication in a reaction mixture with BLUR8 as a template was semiconservative and not primed by a putative RNA polymerase III transcript synthesized on the *Alu* family sequence in vitro. Pulse-chase experiments showed that the small-sized DNA produced in a short-term incubation was converted to full-length closed circular and open circular DNAs in alkaline sucrose gradients. DNA synthesis in extracts began in a region of the *Alu* family sequence and was inhibited 80% by the addition of anti-T serum. Furthermore, partially purified T antigen bound the *Alu* family sequence in BLUR8 by the DNA-binding immunoassay. These results suggest that SV40 T antigen recognizes the *Alu* family sequence, similar to the origin sequence of SV40 DNA, and initiates semiconservative DNA replication in vitro.

A large portion of the DNA from most eucaryotic organisms is organized with alternating regions of unique and repeated sequences; the interspersed repeated sequences are characteristically described as having a size of about 300 base pairs (5, 23). When treated with the restriction endonuclease *Alu*I, these repeated sequences give rise to two fragments, one approximately 170 base pairs and the other approximately 120 base pairs. These repetitive sequences, termed the *Alu* family sequences (10), are estimated to be present approximately 300,000 times, or on the average of once for every few thousand DNA base pairs, throughout the human and Chinese hamster haploid genomes (10, 13, 14).

Jelinek and co-workers have made apparent from nucleic acid sequence studies that there are nucleotide similarities among the *Alu* family of interspersed repeated sequences, regions of cloned DNA fragments containing mRNA coding sequences from both humans and rodents, regions of cloned DNA fragments that can serve as templates in the in vitro RNA polymerase III transcription system, regions in a low-molecular-weight RNA found hydrogen bonded to heterogeneous nuclear RNA from rodent cells, the inverted repeat heterogeneous nuclear RNA from cultured human cells, and a sequence located at one of the junctions between the large intervening sequence and the coding sequence in the human β-globin gene (15). Furthermore, they also showed that these sequences share homology with a sequence at or near the origin of DNA replication in the genome of BK virus, simian virus 40 (SV40), and polyoma virus, suggesting that the *Alu* family of interspersed repeated sequences might function as the origin of DNA replication in mammalian cells (15).

In this study, I examined the template activity of three cloned DNAs containing the *Alu* family and the origins of *Saccharomyces cerevisiae* and *S. cerevisiae* plasmid 2μm DNA in the SV40 DNA replication system. As a result, of the three cloned DNAs, the cloned DNA containing the *Alu* family sequence had good template activity in this SV40 DNA replication system. Replication started at the *Alu* family sequence within this cloned DNA. Therefore, *Alu* family sequences appeared to function as the origin of DNA replication, at least in this system.

MATERIALS AND METHODS

Cells and virus. The SV40-transformed monkey cell line CosI (9) was obtained from Y. Gluzman. FM3A, an established cell line from mouse mammary carcinoma (18), was from T. Enomoto. SV40 was propagated in the monkey cell line GC7, and SV40 DNA was extracted from purified virions by CsCl-ethidium bromide equilibrium centrifugation (29).

Plasmid DNAs. Plasmid BLUR8 containing the human *Alu* family sequence was kindly provided by T. Friedmann (22). YRp7 harboring a yeast autonomous replicating sequence, which was originally constructed by J. Carbon (28), and pJD29 having the origin sequence of yeast plasmid 2μm DNA, which was originally cloned by J. D. Beggs (2), were supplied by M. Yamamoto.

Conditions for in vitro reaction. Nuclear extract and cytoplasm were prepared as described previously (1). HeLa or FM3A nuclei in hypotonic buffer were prepared by Dounce homogenization of cells 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 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 (1). 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. The reaction mixture (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/mmol; 5,000 to 15,000 cpm/pmol), 3.75 mM ATP, 20 μl of HeLa or FM3A nuclear extract (protein concentration, 2.5 mg/ml), 2 μl of CosI cytoplasm (protein concentration, 20 mg/ml), and 0.4 μg of form I SV40 or the cloned DNAs. After incubation of the reaction mixture for 60 min at 37°C and subsequent digestion with proteinase K (10 μg) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol. 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.

DNA-binding immunoassay. Immunoprecipitation of T antigen bound to DNA was performed essentially as described by Prives et al. (19). SV40 T antigen was partially purified from HeLa cells infected with the adenovirus-SV40 recombinant as described previously (1). BLUR8 was digested with *Bam*HI and run on a 1.6% agarose gel containing the same buffer as that for 1% agarose gel. Two fragments (pBR322 and the *Alu* family sequence) were isolated and treated with bacterial alkaline phosphatase, and the 5′ ends were labeled with polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol) to a specific activity of 2×10^4 to 5×10^4 cpm per ng of DNA. After preadjustment of the solution containing 0.3 μg of partially purified T antigen (amount of T antigen, approximately 0.12 μg) to pH 6.8, 5 ng of ^{32}P-labeled DNA fragment and 5 μg of sheared salmon sperm DNA were added, and the reaction as kept at 4°C for 1 h. Anti-SV40 T serum (10 μl) was mixed for an additional 30 min, followed by addition of 25 μl of a 20% suspension of formaldehyde-fixed *Staphylococcus aureus* strain Cowan I in NET buffer (0.15 M NaCl, 10 mM EDTA, 0.05% Nonidet P-40, 10 mM Tris-hydrochloride, pH 7.0). After centrifugation of the immune complexes for 0.5 min at 15,000 rpm, the pellets were washed three times in buffer containing 0.5% Nonidet P-40, 10 mM Tris-hydrochloride (pH 7.5), and 0.15 M NaCl. DNA fragments were dissociated from the immune complex by incubation in 50 μl of TMS buffer (20 mM Tris-hydrochloride, pH 8.5, 0.25 M β-mercaptoethanol, 1% sodium dodecyl sulfate) for 30 min at 37°C. After centrifugation, the pelleted DNA fragments were analyzed by electrophoresis in 1.6% agarose gels.

Materials. Anti-T hamster serum prepared from a tumor-bearing animal was kindly provided by N. Yamaguchi. α-Amanitin was from Sigma Chemical Co. All of the restriction enzymes and polynucleotide kinase were purchased from Takara Shuzo Corp. Klenow DNA polymerase was from Bethesda Research Laboratories. T4 DNA ligase was kindly supplied by S. Sugano. [α-^{32}P]dCTP was from Amersham Corp., and [γ-^{32}P]ATP was from New England Nuclear Corp.

RESULTS

Examination of template activity of cloned DNAs in the SV40 DNA replication system in vitro. The in vitro SV40 DNA replication system, consisting of the nuclear extract from HeLa cells and the cytoplasmic fraction from CosI cells infected with SV40, promotes the initiation and subsequent elongation of DNA replication on the exogenously added SV40 DNA or the cloned DNA containing the origin of SV40 DNA synthesis as a template (1). The reactions in this system are quite similar to those in in vivo SV40 DNA replication: absolute dependence on the large T antigen, which is an early gene product of SV40; specific initiation from a specific site followed by bidirectional replication from the origin; and involvement of DNA polymerase α (1, 12). SV40 DNA resembles a replicon in the eucaryotic genome in terms of the shape and structure of DNA and the mode of DNA replication. Therefore, it seems that this system could be a good assay system for understanding the molecular mechanism of DNA synthesis in higher organisms.

The original system we developed consisted of mixed extracts from uninfected HeLa nuclei and SV40-infected CosI cytoplasm. HeLa nuclear extract can be replaced by the extract of FM3A, an established cell line from mouse mammary carcinoma (18). Therefore, I used both nuclear extracts in these experiments.

The template activity of five DNAs, including SV40, BLUR8 (human *Alu* family), pJD29 (2μm DNA), YRp7 (yeast autonomous replicating sequence), and pBR322 which was the BLUR8 plasmid in the absence of the *Alu* family sequence, was examined in the in vitro SV40 replication system, and the products were run on neutral agarose gels. The distribution patterns of labeled DNA after autoradiography are shown in Fig. 1. SV40 DNA was a good template and

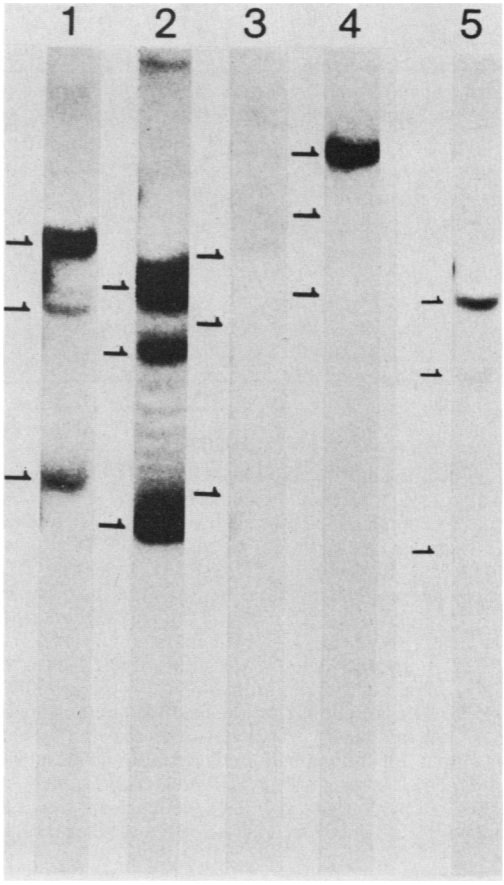

FIG. 1. DNA synthesis in vitro on various cloned DNAs. Synthesis was carried out with 0.4 μg of each DNA as a template. After 60 min at 37°C, the reaction mixture was treated with pronase and sodium dodecyl sulfate, and the DNA was precipitated with ethanol. DNA was visualized by autoradiography after agarose gel electrophoresis. (Lane 1) Reaction with SV40 DNA as a template; (lane 2) BLUR8; (lane 3) YRp7; (lane 4) pJD29; (lane 5) pBR322. Total incorporated dCMP amounts estimated by counting of a 0.1-ml sample of the mixture were 2.1, 4.5, 0.1, 0.8, and 0.2 pmol per μg of added DNA in lanes 1 to 5, respectively. The horizontal bars represent the positions of form II, III, and I DNA from the top.

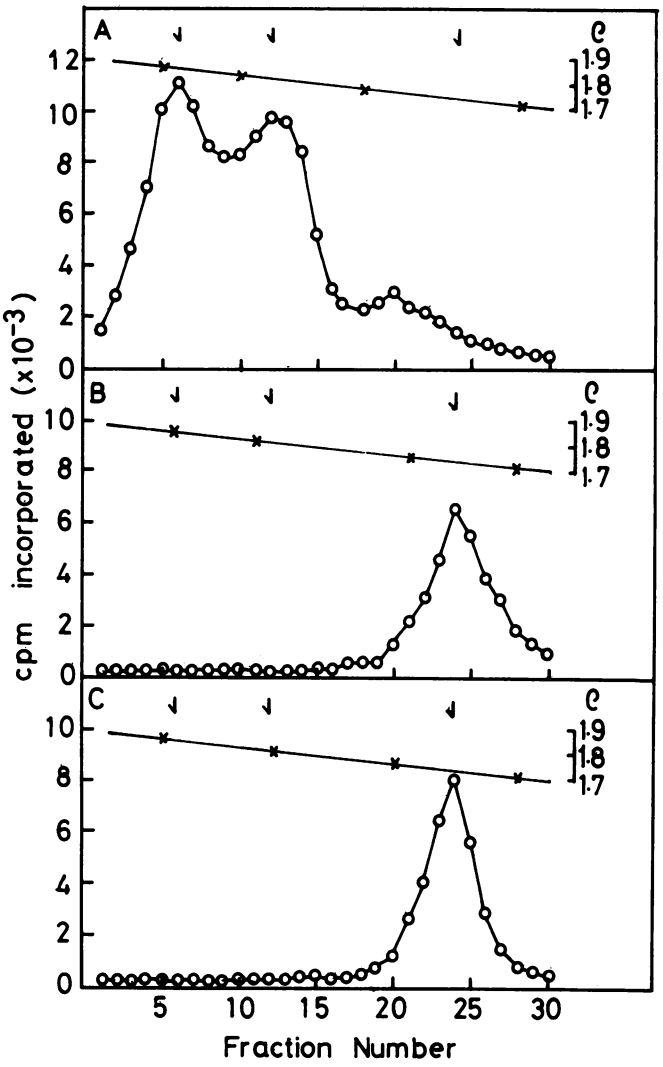

FIG. 2. Isopycnic centrifugation at neutral pH of DNA synthesized in vitro in the presence of deoxybromo-UTP. A standard reaction mixture containing 30 μM each dATP, dGTP, and deoxybromo-UTP (in place of dTTP) and 1.5 μM [^{32}P]dCTP was carried out on BLUR8 (A), YRp7 (B), or pJD29 (C) for 60 min at 37°C. The DNA was extracted with phenol after treatment of the mixture with proteinase K (500 μg/ml) for 20 min at 37°C and precipitated with ethanol. The density of the DNA sample containing 25 mM Tris (pH 7.4), 1 mM EDTA, and 0.1 M NaCl was adjusted to 1.8 g/cm^3 by the addition of CsCl and centrifuged in an SW50.1 rotor at 43,000 rpm for 48 h at 25°C. After fractionation, the acid-insoluble radioactivity was counted, and the density was measured. Arrows indicate the expected positions for fully substituted, hybrid, and unsubstituted DNAs (left to right, respectively). Total incorporated dCMP amounts estimated by counting 0.1 ml of the reaction were 3.8, 0.8, and 0.9 pmol per μg of added DNA in (A), (B), and (C), respectively.

gave rise to three major bands, forms II, III, and I from the top of the gel, as previously described (1) (Fig. 1, lane 1). pJD29, YRp7, and pBR322, however, all gave rise to form II or III (Fig. 1, lanes 3, 4, and 5), which were derived from the repair reaction, but not from semiconservative replication as shown later (Fig. 2). With BLUR8 as a template in the reaction, somewhat different results were obtained (Fig. 1, lane 2). The products were forms I, II, and III, and other forms consisted of closed circular DNA with different numbers of superhelical turns which could be converted to linear form III by *Eco*RI digestion. Almost all of the DNAs synthesized with BLUR8 as a template were from semiconservative replication (see Fig. 2).

Evidence for semiconservative replication. To determine whether in vitro products were produced by semiconservative replication or repair synthesis, the DNAs were synthesized in a reaction mixture containing bromodeoxy-UTP in place of dTTP and analyzed by neutral CsCl equilibrium centrifugation (Fig. 2). When BLUR8 was used as a template in the reaction, approximately 43% of the in vitro products were fully substituted by bromouracil, 13% were intermediate between fully and half substituted, 37% banded at a hybrid density which was half substituted, and only 7% of the product had unsubstituted density (Fig. 2A). This result clearly indicates that approximately 93% of the in vitro products in the reaction with BLUR8 as a template were derived from semiconservative DNA replication. On the other hand, when the reaction was carried out with YRp7 or pJD29 as template, almost all of the products banded at densities unsubstituted or slightly greater than unsubstituted DNA (Fig. 2 and C), indicating that the reaction with YRp7 or pJD29 as template was the repair-type reaction. When the same experiment was performed after the reaction with pBR322 as template, the same results as those with YRp7 and pJD29 were obtained (data not shown). I would say that form I DNA observed on a neutral agarose gel after the reaction is the typical sign that DNA synthesis was performed semiconservatively. Therefore, the experiments have been done in detail with BLUR8 as a template in the reaction.

Effect of α-amanitin on the reaction. There is a possibility that some small-sized RNAs synthesized in this reaction mixture with BLUR8 as a template were used in the DNA replication reaction shown here as a primer because *Alu* family clones could serve as templates in the in vitro RNA polymerase III transcription system (7, 15). To clarify this point, α-amanitin, which is the specific inhibitor of RNA polymerases II and III (24), was added to the reaction mixture (Table 1). No or little effect on the DNA-synthesizing reaction was obtained with the concentrations used, whereas >90% of the RNA-synthesizing reaction was inhibited at a concentration of >100 μg/ml. These results indicate that DNA synthesis observed in this reaction mixture cannot be primed by RNA polymerase transcripts.

Size distribution of BLUR8 DNA synthesized in vitro. BLUR8 DNA synthesized in vitro was analyzed by sedimentation through alkaline sucrose gradients (Fig. 3). When the reaction was carried out for 5 min, almost all of the DNA synthesized was of the small size which seemed to be putative Okazaki fragments (Fig. 3A). After 60 min of

TABLE 1. Effect of α-amanitin on BLUR8 reaction[a]

α-Amanitin concn (μg/ml)	[^{32}P]dCMP incorporation (pmol/μg of DNA)	[^{32}P]rUMP incorporation (pmol/μg of DNA)
0	3.32	0.31
1	3.65	0.26
10	3.06	0.21
50	3.03	0.12
100	3.33	0.03
200	3.10	0.02

[a] All reactions were carried out with 0.4 μg of BLUR8 in the presence of the various concentrations of α-amanitin. In the case of the RNA polymerase reaction, 200 μM each rGTP and rCTP and 20 μM [α-^{32}P]rUTP (410 Ci/mmol; 10,000 cpm/pmol) were also added to the DNA synthesis reaction lacking [α-^{32}P]dCTP. After 60 min at 37°C, the acid-insoluble radioactivity was counted.

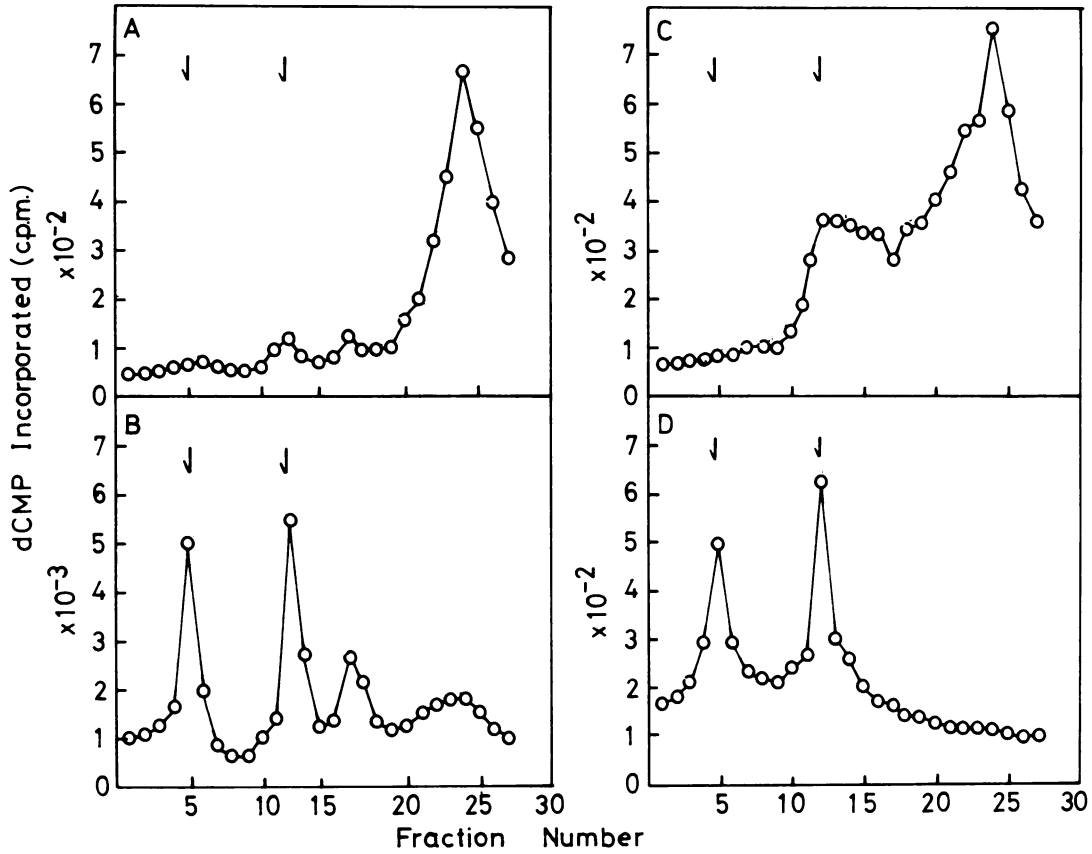

FIG. 3. Sedimentation of the in vitro product in an alkaline sucrose gradient. After the reaction on BLUR8, DNA was extracted with phenol and precipitated with ethanol. The samples were loaded onto 5 to 20% alkaline sucrose gradients containing 0.2 M NaOH, 0.5 M NaCl, and 10 mM EDTA and centrifuged in an SW50.1 rotor at 45,000 rpm for 3 h at 4°C. The acid-insoluble radioactivity was counted after fractionation. (A) Reaction for 5 min. (B) Reaction for 60 min continuously. (C) Reaction for 5.5 min. (D) After the reaction for 5.5 min, a 200-fold concentration of cold dCTP (300 μM) was added to the mixture, and the reaction was continued for up to 60 min. Arrows show the positions of the denatured supercoiled and open circular BLUR8 DNAs. Under these conditions denatured open circular and linear DNAs formed a single peak. Total incorporated dCMP amounts estimated by counting 0.1 ml of the reaction were 0.5, 5.2, 0.7, and 0.7 pmol per μg of added DNA in (A), (B), and (C), respectively.

incubation, however, the two major peaks corresponding to denatured supercoils and open circles, in addition to the small-sized DNA, could be seen (Fig. 3B). Similar distribution patterns were also observed in pulse-chase experiments (Fig. 3C and D). After a 5.5-min pulse of the mixture (Fig. 3C), incubation was continued for a further 60 min in the presence of a 200-fold excess of cold dCTP. From a comparison of the pulse and chase samples, it is clear that the DNAs smaller than the single-stranded circular DNA were converted to the denatured supercoiled and single-stranded circular DNAs. These are typical replication patterns observed in vivo and in vitro with double-stranded circular DNAs such as SV40 (16, 27; H. Ariga, submitted for publication).

Determination of the initiation point in the reaction. DNA synthesis carried out in a mixture of HeLa nuclear extract and SV40-infected CosI cytoplasm starts at the origin-containing region of SV40 DNA and proceeds bidirectionally (1). To assess this point in the reaction with BLUR8 as template, newly synthesized DNA labeled for 10 and 60 min was extracted and digested with *Bam*HI, and the distribution of radioactivity on each fragment was examined by agarose gel electrophoresis (Table 2). Digestion of DNA with *Bam*HI yielded two fragments, the sizes of which were 270 and 4,362 base pairs. The small fragment contains the *Alu* family sequence. The ratio of the radioactivity of the small fragment to the large fragment was normalized to the ratio of the nucleotide numbers of two fragments (1:16.16). Therefore, the value 1 means that all of the sequences are equally labeled. In the first 10 min of the reaction, a very high ratio (18.74) was obtained. After 60 min, the ratio decreased to 1.43, indicating that the label became more evenly incorporated on the two fragments. These results clearly show that

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

Time (min)	cpm incorporated		Ratio small/large	Normalized ratio
	Small-fragment	Large-fragment		
10	406	350	1.16	18.74
60	2,530	28,540	0.09	1.43

[a] The in vitro reaction was carried out with 0.2 μg of BLUR8. After incubation of the reaction mixture for 10 or 60 min at 37°C and subsequent digestion with pronase and sodium dodecyl sulfate for 20 min at 37°C, the DNA was extracted with phenol saturated with 10 mM Tris (pH 8.1)–1 mM EDTA and precipitated with ethanol. The DNA was dissolved in water, mixed with 1.5 μg of cold BLUR8, digested with *Bam*HI, and electrophoresed on a 2% agarose gel containing 40 mM Tris (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 (small fragment/large fragment) was normalized to the ratio of the nucleotide numbers of two fragments. The nucleotide numbers of large and small fragments are 4,362 and 270, respectively (ratio, 16.16).

DNA synthesis starts at the *Alu* family sequence in the reaction containing the SV40-infected CosI cytoplasm and HeLa nuclear extract.

Effect of anti-T serum on the reaction. SV40 T antigen is definitely necessary for the in vitro SV40 DNA replication system used here, as reported previously (1). To examine whether the same case occurs in the reaction with BLUR8 as template, anti-T serum was added to the mixture and the products were visualized by autoradiography after neutral agarose gel electrophoresis (Fig. 4). This serum was clearly specific for the T antigen based on immunoprecipitation experiments with SV40-infected CV1 cell extract. The amount of incorporation after the addition of anti-T serum was generally reduced in all bands (lane 2). Approximately 20% of the incorporation was recovered compared with that without addition of anti-T serum. Of the three bands corresponding to forms I, II, and III of BLUR8 DNA, the greatest reduction occurred in form I DNA. In contrast, there was little change in the pattern of the product after the addition of normal hamster serum (lane 3) as compared with that of the serum-minus control (lane 1). These results suggest that

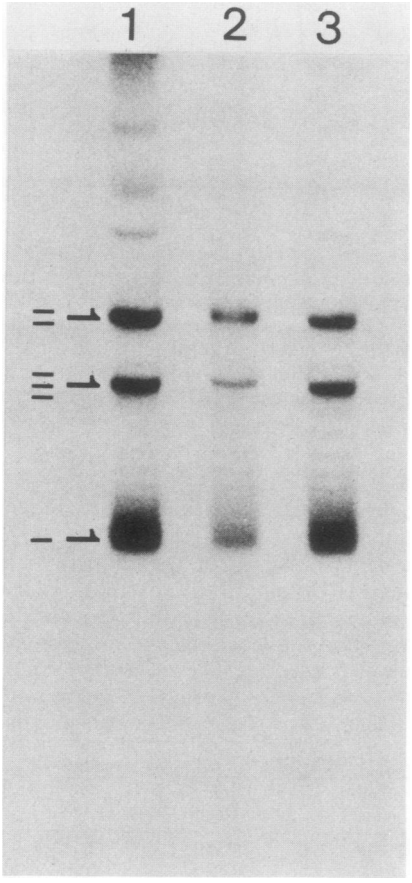

FIG. 4. Effect of anti-T serum on the reaction. All reactions were carried out with 0.4 μg of BLUR8. When serum (10 μl) was added to the reaction, the mixtures were held for 10 min at 4°C before synthesis was started at 37°C. After 60 min at 37°C, the DNA was processed from all reaction mixtures as in the legend to Fig. 1. (Lane 1) Control reaction, no addition of serum; (lane 2) plus anti-T hamster serum; (lane 3) plus normal hamster serum. Total incorporated dCMP amounts estimated by counting a 0.1-ml sample of the mixture were 3.5, 0.2, and 3.1 pmol per μg of added DNA in lanes 1 to 3, respectively. Horizontal bars represent the positions of form II, III, and I BLUR8 DNA.

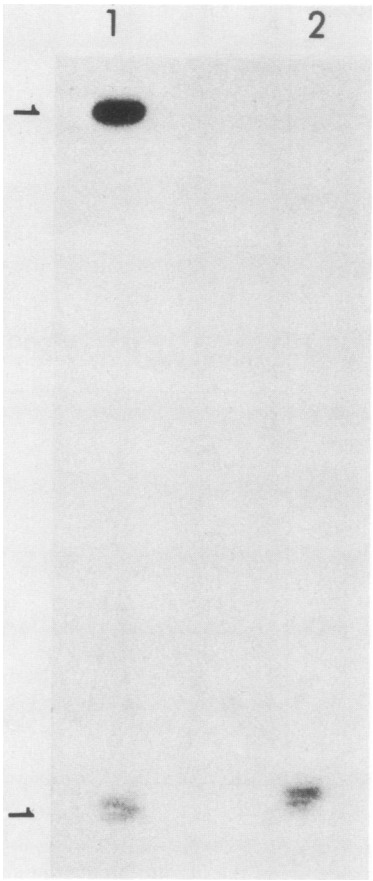

FIG. 5. Binding of T antigen to DNA fragments of BLUR8. A 0.3-μg portion of extracted proteins containing T antigen (amount of T antigen, approximately 0.12 μg) was bound to 5 ng of ^{32}P-labeled *Bam*HI BLUR8 fragments plus 5 μg of sheared salmon sperm DNA, followed by immunoprecipitation with anti-SV40 T serum. Bound DNA was released and analyzed by agarose gel electrophoresis and autoradiography. (Lane 1) Input DNAs without any reaction; (lane 2) bound DNA after the reaction. Arrows represent the *Bam* fragments which are linear pBR322 and *Alu* family sequence DNAs (from the top, respectively).

SV40 T antigen may recognize the sequence of BLUR8 DNA and promote the initiation of DNA synthesis as is the case with SV40 DNA.

***Alu* family DNA binding of T antigen.** The best-studied biochemical property of T antigen linked to its role in viral DNA replication is its specific affinity for sequences at the replication origin. A DNA-binding immunoassay was developed recently (19), making it possible to analyze the origin-binding activity of T antigen. The result presented in Fig. 4 suggests the involvement of T antigen in DNA synthesis produced in a reaction mixture with BLUR8 as template. Therefore, the binding activity of T antigen to BLUR8 was examined by the same immunoassay as for SV40 DNA (Fig. 5). Partially purified SV40 T antigen from HeLa cells infected with an adenovirus-SV40 recombinant producing the authentic T antigen (1) was bound to ^{32}P-labeled BLUR8 fragments under suitable conditions and immunoprecipitated with anti-T-antigen antibody. Analysis of the labeled DNA fragments released from the immune complexes showed that the fragment containing the *Alu* family sequence was preferentially bound with T antigen (Fig. 5, lane 2). These data clearly show that SV40 T antigen recognizes and binds the

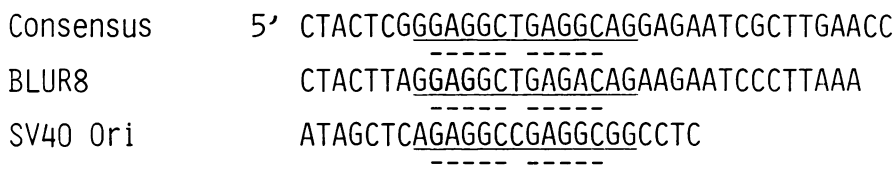

FIG. 6. Sequence comparison of portions of three DNAs: (top) consensus sequence of the human *Alu* family of interspersed repeated DNA; (middle) clone of one member of the human *Alu* family, BLUR8; (bottom) region near the origin of replication of SV40 DNA. The sources for these sequence determinations are references 10, 22, and 8 and 21, respectively, and were summarized by Jelinek et al. (15). The solid line shows sequence homologies emphasized in the text common to the sequences given. The dashed lines indicate the putative T-antigen-binding sequences described by Tegtmeyer et al. (25). All sequences are written with the 5' side on the left and the 3' side on the right.

Alu family sequence to promote the initiation of DNA replication in vitro as in the case of SV40 DNA. However, I could not rule out the possibility that T antigen-like proteins or other proteins present in HeLa cells bind the *Alu* family sequence.

DISCUSSION

This paper shows that the initiation of DNA replication may occur on a cloned DNA containing the human *Alu* family sequence in a soluble extract system promoting SV40 DNA replication, as described previously (1). This system, which consists of uninfected HeLa or FM3A nuclear extract and SV40-infected CosI cytoplasm, depends on the presence of SV40 T antigen and its binding site II in the template DNA, and replication starts at the origin of SV40 DNA synthesis. When one of the clones of the human *Alu* family sequence, BLUR8, was used as a template in this SV40 system, similar results were obtained: initiation of replication in the *Alu* family containing fragment and dependence on SV40 T antigen.

The biological function of the *Alu* family sequence is unknown. Jelinek et al. have noted sequence similarities between selected portions of the *Alu* family and several other RNA or DNA sequences which are known or suspected to be involved in DNA replication, transcription control, and mRNA processing (15). The nucleotide sequences of a portion of BLUR8 and an origin region of SV40 DNA are quite similar but not identical (Fig. 6). The SV40 sequence shown here is half of T antigen binding site II, 5'AGAGGCCGAGGCGGCCTCGGCCTCTG3', as determined by Tjian (26). The sequence in BLUR8 (Fig. 6) is similar to the SV40 sequence shown above. Tegtmeyer and his colleagues found that T antigen binds the consensus sequence 5'-(G>T) (A>G)GGC-3' which is present in BLUR8 (Fig. 6) (25). Therefore, it is likely that SV40 T antigen recognizes the SV40-like sequence in BLUR8 and promotes the initiation of replication by using the same mechanism as that for SV40. Indeed, anti-T serum inhibited DNA replication to a large extent, and T antigen also binds the *Alu* family sequence. On the contrary, the clone containing the yeast autonomous replicating sequence or the origin sequence of 2μm DNA had no template activity in this SV40 system. There are no sequence similarities among the autonomous replicating sequence, the origin of 2μm DNA, and SV40 DNA (2, 28).

McCutchan and Singer reported the molecular cloning of African green monkey genomic DNA segments that hybridize to the region around the origin of replication of SV40 (17, 20). They showed that these cloned DNAs contain a large number of short stretches homologous to three specific noncoding domains around the SV40 origin of replication, including the 27-base pair region of dyad symmetry in which T antigen binding site II exists.

Dhruva et al. (6) isolated the spontaneously formed viable mutant of SV40 carrying a DNA insert that is presumably of host (monkey) cell origin. The sequence of the insert bore a close resemblance to a certain predominant family of interspersed repeated sequences of monkey genome which is homologous to the human *Alu* family (6). Conrad and Botchan isolated the cloned DNAs homologous to the SV40 origin region from the human genomic library (3). The homologous sequences in these clones were not members of the *Alu* family of repeats and hybridized to SV40 DNA more strongly than do *Alu* family members (3). Therefore, I cannot clearly demonstrate from the experiments described here that the *Alu* family sequences are the initiation site of DNA replication as suggested by Jelinek et al. (15). To clarify this point, experiments to identify the first nucleotide incorporated and the precise location of the binding site for SV40 T antigen in this in vitro system should be done.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Education, Science and Culture, Japan.

I am grateful to M. Matsumoto and Y. Mizusawa for beautiful typing of the manuscript, and I am indebted to N. Yamaguchi and S. Sugano for the critical reading of the manuscript.

LITERATURE CITED

1. **Ariga, H., and S. Sugano.** 1983. Initiation of simian virus 40 DNA replication in vitro. J. Virol. **48**:481–491.
2. **Beggs, J. D.** 1978. Transformation of yeast by a replicating hybrid plasmid. Nature (London) **275**:104–109.
3. **Conrad, S. E., and M. R. Botchan.** 1982. Isolation and characterization of human DNA fragments with nucleotide sequence homologies with the simian virus 40 regulatory region. Mol. Cell. Biol. **2**:949–965.
4. **Deininger, P. L., D. J. Jolly, C. M. Rubin, T. Friedman, and C. W. Schmid.** 1981. Base sequence studies of 300 nucleotide renatured repeated human DNA clones. J. Mol. Biol. **151**:17–33.
5. **Deininger, P. L., and C. W. Schmid.** 1976. An electron microscope study of the DNA sequence organization of the human genome. J. Mol. Biol. **106**:773–790.
6. **Dhruva, B. R., T. Shenk, and K. N. Subramanian.** 1980. Integration *in vivo* into simian virus 40 DNA of a sequence that resembles a certain family of genomic interspersed repeated sequence. Proc. Natl. Acad. Sci. U.S.A. **77**:4514–4518.
7. **Duncan, C. H., P. Jagadeeswaran, R. R. C. Wang, and S. M. Weissman.** 1981. Structural analysis of templates and RNA polymerase III transcripts of *Alu* family sequences interspersed among the human β-like globin genes. Gene **13**:185–196.
8. **Fiers, W., R. Contreras, G. Halgeman, R. Rogiers, A. van de Voorde, H. van Heuverswyn, J. van Herreweghe, G. Volchaert, and M. Ysebaert.** 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) **293**:113–120.
9. **Gluzman, Y., R. J. Frisque, and J. Sambrook.** 1980. Origin-defective mutant of SV40. Cold Spring Harbor Symp. Quant. Biol. **44**:293–300.
10. **Houck, C. M., F. P. Rinehart, and C. W. Schmid.** 1979. An ubiquitous family of repeated DNA sequences in the human

genome. J. Mol. Biol. **132**:289–306.
11. **Hyynes, S. R., and W. R. Jelinek.** 1981. Low molecular weight RNAs transcribed *in vitro* by RNA polymerase III from *Alu*-type dispersed repeats in Chinese hamster DNA are also found in vivo. Proc. Natl. Acad. Sci. U.S.A. **78**:6130–6134.
12. **Ikegami, S., T. Taguchi, M. Oguro, H. Nagano, and Y. Mano.** 1978. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-α. Nature (London) **275**:458–460.
13. **Jelinek, W. R.** 1977. Specific nucleotide sequences in HeLa cell inverted repeated DNA: enrichment for sequences found in double-stranded regions of heterogeneous nuclear RNA. J. Mol. Biol. **115**:591–601.
14. **Jelinek, W. R.** 1978. Inverted repeated DNA from Chinese hamster ovary cells studies with cloned DNA fragments. Proc. Natl. Acad. Sci. U.S.A. **75**:2679–2683.
15. **Jelinek, W. R., T. P. Toomey, L. Leinward, C. H. Duncan, P. A. Biro, P. V. Chouday, S. M. Weissman, C. M. Rubin, C. M. Houck, P. L. Deininger, and C. W. Schmid.** 1980. Ubiquitous, interspersed repeated sequences in mammalian genome. Proc. Natl. Acad. Sci. U.S.A. **77**:1398–1402.
16. **Kornberg, A.** 1980. DNA replication. W. H. Freeman, San Francisco.
17. **McCutchan, T. F., and M. F. Singer.** 1981. DNA sequences similar to those around the simian virus 40 origin of replication are present in the monkey genome. Proc. Natl. Acad. Sci. U.S.A. **78**:95–99.
18. **Nakano, N.** 1966. Establishment of cell lines *in vitro* from a mammary ascites tumor of mouse and biological properties of the established lines in a serum containing medium. Tohoku J. Exp. Med. **88**:69–84.
19. **Prives, C., L. Covey, A. Scheller, and Y. Gluzman.** 1983. DNA binding properties of simian virus 40 T-antigen mutants defective in viral DNA replication. Mol. Cell. Biol. **3**:1958–1966.
20. **Queen, C., S. T. Lord, T. F. McCutchan, and M. F. Singer.** 1981. Three segments from the monkey genome that hybridize to simian virus 40 have common structural elements. Mol. Cell. Biol. **1**:1061–1068.
21. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–502.
22. **Rubin, C. M., C. M. Houck, P. L. Deininger, T. Friedmann, and C. W. Schmid.** 1980. Partial nucleotide sequence of the 300-nucleotide interspersed repeated human DNA sequences. Nature (London) **284**:372–374.
23. **Schmid, C. W., and P. L. Deininger.** 1975. Sequence organization of the human genome. Cell **6**:345–358.
24. **Schwartz, L. B., V. E. F. Sklar, J. A. Jaehning, R. Weinman, and R. G. Raeder.** 1974. Isolation and partial characterization of the multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in the mouse myeloma, MOPC 315. J. Biol. Chem. **249**:5889–5897.
25. **Tegtmeyer, P., B. A. Lewton, A. L. DeLucia, V. G. Wilson, and K. Ryder.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of protein bound to the origin of replication. J. Virol. **46**:151–161.
26. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen related protein. Cell **12**:165–179.
27. **Tooze, J.** 1980. DNA tumor viruses: molecular biology of tumor viruses, part 2, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Tschumper, C., and J. Carbon.** 1980. Sequence of a yeast DNA fragment containing a chromosomal replicator and the *TRP1* gene. Gene **10**:157–166.
29. **Yamaguchi, N., and T. Kuchino.** 1975. Temperature-sensitive mutants of simian virus 40 selected by transforming ability. J. Virol. **15**:1297–1301.