

Cloned Mouse DNA Fragments Can Replicate in a Simian Virus 40 T Antigen-Dependent System In Vivo and In Vitro

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Mouse liver DNA was cut out with *Bam*HI and cloned into YIp5, which contained the URA3 gene of *Saccharomyces cerevisiae* in pBR322. Of the several plasmids isolated, two plasmids, pMU65 and pMU111, could transform *S. cerevisiae* from the URA⁻ to the URA⁺ phenotype and could replicate autonomously within the transformant, indicating that mouse DNA fragments present in pMU65 or pMU111 contain autonomously replicating sequences (ARS) for replication in *S. cerevisiae*. Furthermore, to determine the correlation between ARS function in yeast cells and that in much higher organisms, we tried to challenge these plasmids with the simian virus 40 (SV40) DNA replication system. Of the two plasmids tested, the *Eco*RI-*Bgl*II region of pMU65 could be hybridized with a chemically synthesized 13-nucleotide fragment corresponding to the origin region of SV40 DNA. Both pMU65 (the *Eco*RI-*Bgl*II region cloned in pBR322) and its subclone pMU65EB could replicate semiconservatively, and initiation of DNA replication started from the *Eco*RI-*Bgl*II region when the replicating activity of these plasmids was tested in the in vitro SV40 DNA replication system we have established before. Furthermore, pMU65 and pMU65EB could replicate autonomously within monkey Cos cells which produce SV40 T antigen constitutively. These results show that a 2.5-kilobase fragment of the *Eco*RI-*Bgl*II region in pMU65 contains the ARS needed for replication in the SV40 DNA replication system.

The replication of eucaryotic DNA occurs by initiation at many chromosomal sites which are usually clustered in a specific region characteristic of the cell type and the stage of the cell cycle (7, 9, 12, 19, 43). Recently, several investigators have tried to isolate replication origins by using in vitro recombination techniques (1, 16, 21, 41). Another approach to isolation of the replication origin is to get autonomous replicating sequences (ARS). Many eucaryotic ARS of yeasts, as well as those of other higher organisms, have been described (8, 10, 14, 18, 20-22, 24, 26, 28, 29, 33, 34, 37-40, 44, 45; J. M. Vallet, M. Rahire, and J. D. Rochaix, EMBO J., in press). These sequences from higher organisms may act as initiators of replication in yeast cells. However, there is no evidence that these cloned eucaryotic DNA segments can also act as replicators in cells of the original higher organism. Another approach to finding the replication origin is to search for the sequences similar to the origin of the eucaryotic virus genome, because the regulatory sequences might be conserved over the species (25). Simian virus 40 (SV40) DNA seems to resemble one replicon of the eucaryotic genome. Therefore, several investigators tried to find, in monkey or human cells, sequences similar to that of the SV40 origin (11, 27, 30). We have already developed the in vitro system in which initiation of DNA replication occurs on exogenous DNA containing the SV40 origin (4). In this system, the human *Alu* family, which is one of interspersed repeated sequences (35), showed a good template activity, and the initiation started from *Alu* family sequences (2).

Now we have tried to search for sequences from several genomic clones which are similar to the SV40 origin sequence by using an in vitro SV40 DNA replication system and hybridization technique. To determine the correlation of the ARS in yeasts and much higher organisms, we first screened C3H mouse genomic fragments which could repli-

cate in yeast cells. Of the three DNA clones tested, a 2.5-kilobase (kb) fragment from only one clone hybridized to half of a 27-nucleotide perfect palindrome present in the SV40 origin. This original clone replicated in yeast cells, its 2.5-kb fragment had a good template activity when cloned in pBR322, and the initiation of DNA replication started from the region of the 2.5-kb fragment in an in vitro system. Furthermore, these two clones could replicate autonomously in the nuclei of SV40-transformed Cos cells, producing SV40 T antigen constitutively when clones were transfected onto Cos cells.

MATERIALS AND METHODS

Strains. *Saccharomyces cerevisiae* YNN27 (*trp1-289 ura3-52 gal2*) cells were grown in YEPD medium (1% yeast extract, 2% polypeptone, 2% glucose; pH 5.3). Monkey Cos-I cells were grown in Dulbecco modified Eagle medium containing 10% newborn calf serum.

Conditions for in vitro reaction. HeLa nuclear extract and SV40-infected Cos-I cytoplasm were prepared as described previously (4). The reaction mixture (100 μ l) contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5]), 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dTTP, 1.5 μ M [α -³²P]dCTP (410 Ci/mmol; 5,000 to 15,000 cpm/pmol), 3.75 mM ATP, 20 μ l of HeLa nuclear extract (protein concentration, 2.5 mg/ml), 2 μ l of Cos-I cytoplasm (protein concentration, 20 mg/ml), and 0.4 μ g of form I cloned DNAs. After incubation of the reaction mixture for 60 min at 37°C and subsequent digestion with proteinase K (10 μ g) and sodium dodecyl sulfate (0.2%) for 20 min at 37°C, the DNA was precipitated with ethanol. The DNA was dissolved in 50 μ l 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 XRS X-ray film.

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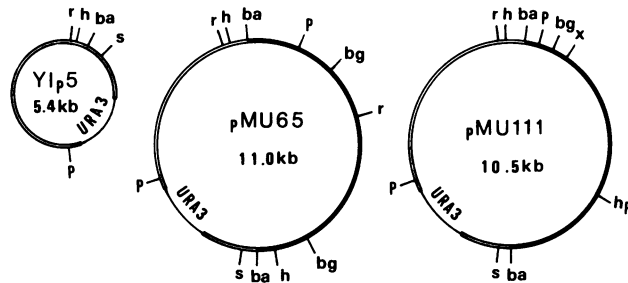


FIG. 1. Restriction maps of plasmids used in this study. Maps of YIp5, pMU65, and pMU111 were drawn. Abbreviations and symbols: ba, *Bam*HI; r, *Eco*RI; s, *Sal*I; x, *Xho*I; hp, *Hpa*I; h, *Hind*III; p, *Pvu*II; bg, *Bgl*II; —, yeast *URA3* gene; □, pBR322; ■, mouse DNA.

Replication of plasmid DNA in *Cos* cells. To check for plasmid DNA replication, *Cos*-I cells were transfected with 0.5 μ g of plasmid DNA by a calcium phosphate precipitation technique (42). After 18 h, fresh medium was added to the plate, and the cells were maintained at 37°C for a further 48 h unless otherwise indicated. The cells were then harvested and extracted by the Hirt procedure (17). The low-molecular-weight DNA in the Hirt supernatant was deproteinized with 100 μ g of proteinase K per ml at 37°C for 20 min, extracted twice with phenol, and precipitated with ethanol. The DNA preparation was digested with 100 μ g of ribonuclease A per ml at 37°C for 20 min, fractionated by 1% agarose gel electrophoresis, transferred to nitrocellulose paper (36), and hybridized to a ³²P-labeled pBR322 probe (32). The hybridization was carried out in a mixture containing 5 \times SSPE (1 \times SSPE contains 0.18 M NaCl, 10 mM NaPO₄ [pH 7.7], and 1 mM EDTA), 2 \times Denhardt solution (1 \times Denhardt contains 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 50 μ g of heat-denatured salmon testis DNA per ml, and ³²P-labeled pBR322. After 16 h at 65°C, the filter was washed twice with 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% sodium dodecyl sulfate and then twice with 0.1 \times SSC–0.1% sodium dodecyl sulfate and then autoradiographed at –80°C.

RESULTS

Isolation of the mouse DNA fragments which replicate autonomously in *S. cerevisiae*. To obtain mouse DNA sequences that function as ARS in yeast cells, we used the method described by Stinchcomb et al. (38). The DNAs from mouse liver nuclei were completely digested with *Bam*HI, and DNA fragments were then cloned into the *Bam*HI site of YIp5, which contains the *URA3* gene of *S. cerevisiae* cloned in pBR322 (Fig. 1). *S. cerevisiae* YNN27 was transformed with plasmid DNAs from a *URA*[–] to a *URA*⁺ phenotype. Transformants appeared on selective plates and were counted 4 or 5 days after transfection. We found that two of these clones, pMU65 and pMU111, could transform strain YNN27 to high efficiency (350 and 320 transformed colonies per μ g of DNA, respectively), whereas YIp5 (less than 0.3 colonies) and other clones (pMU115, 0.3 colonies) could not. Properties of these two clones were in agreement with the results described by Roth et al. (34): (i) the doubling time of each transformant was longer in selective medium than in non-selective medium, (ii) the *URA*⁺ phenotype of the transformants was unstable both in selective and nonselective media, and (iii) the original plasmid could be recovered from transformants of *Escherichia coli* X1176 transformed with

DNAs extracted from the yeast transformants (data not shown). These results clearly show that pMU65 and pMU111 contain ARS which function in yeast cells. Restriction maps of these two clones were determined (Fig. 1). These restriction maps are different from that of mouse mitochondria DNA (34), and the hybridization experiments with mouse DNA and pMU65 or pMU111 clearly showed that these DNAs originated from chromosomal DNA. Next, the forms of plasmid DNAs in yeast transformants were analyzed by Southern blot hybridization. The DNAs extracted from transformants grown in liquid selective media were electrophoresed on an 0.8% agarose gel with or without digestion by *Sal*I. Both pMU65 and pMU111 have one *Sal*I site (Fig. 1). The DNAs were then transferred to a nitrocellulose filter and hybridized with ³²P-labeled pBR322 (Fig. 2). When DNAs were not digested with *Sal*I, both DNAs gave two hybridized bands corresponding to forms I and II of the original plasmids. When cut with *Sal*I, both DNAs gave one hybridized band corresponding to the linear form of the original plasmid. These results clearly show that plasmid DNAs exist extrachromosomally in the transformants of *S. cerevisiae*.

Identification of the fragments which hybridize to SV40 origin sequences. To search for the mouse DNA sequences similar to SV40 origin sequences, we chemically synthesized 13 nucleotides, AGAGGCCGAGGCG, corresponding to half of a 27-base-pair perfect palindrome present in the SV40 origin (13, 31). pMU65 and pMU111 DNAs were digested with several restriction enzymes and fractionated on agarose gels. DNA bands were transferred to nitrocellulose paper and hybridized to the 5' ³²P-labeled 13-nucleotide probe (Fig. 3). Of the 5.9 kb of mouse DNA in pMU65, only 3.4 kb of the *Bgl*II–*Bgl*II fragment (lane 1) and 2.5 kb of the *Eco*RI–*Bgl*II fragment (lane 2) were hybridized to the 13 ³²P-labeled nucleotides, indicating that sequences exist that are similar to those of SV40 origin in 2.5 kb of the *Eco*RI–*Bgl*II fragment of pMU65 DNA. Therefore, 2.5 kb of the *Eco*RI–*Bgl*II fragment from pMU65 was further cloned into *Eco*RI–*Bam*HI sites of pBR322 (called pMU65EB) and used for the subsequent experiments. On the other hand, none of the fragments derived from pMU111 were hybridized to the 13 ³²P-labeled nucleotides (Fig. 3, lanes 3 and 4), suggesting that there was no homology between mouse DNA sequences

TABLE 1. Identification of the initiation point of the reaction^a

Template DNA	Frag-ment	Length (kb)	cpm incorpo-rated		Ratio		Normalized ratio	
			5 min	60 min	5 min	60 min	5 min	60 min
pMU65	A	6.05	452	5,052	1.00	1.00	1.00	1.00
	B	2.50	1,349	3,183	2.98	0.63	7.22	1.52
	C	1.80	163	1,818	0.36	0.36	1.20	1.20
	D	0.95	249	960	0.55	0.19	3.51	1.20
pMU111	A	5.48	1,050	3,538	1.00	1.00	1.00	1.00
	B	3.06	714	2,229	0.68	0.63	1.21	1.12
	C	1.66	357	1,230	0.34	0.34	1.11	1.13

^a The in vitro reaction was carried out with 0.4 μ g of pMU65 or pMU111. After incubation of the reaction mixture for 5 or 60 min at 37°C and subsequent digestion with pronase and sodium dodecyl sulfate for 20 min at 37°C, the DNAs were extracted with phenol, saturated with 10 mM Tris (pH 8.1)–1 mM EDTA, and precipitated with ethanol. The DNAs were dissolved in water, mixed with 1.5 μ g of cold pMU65 or pMU111, and digested with *Eco*RI and *Bgl*II (pMU65) or *Bam*HI and *Hpa*I (pMU111). DNA fragments were then separated on a 1% agarose gel containing 0.5 μ g of ethidium bromide per ml and cut out after illumination under UV light, and the radioactivity was counted.

from pMU111 and SV40 origin sequences under these experimental conditions. Furthermore, these results also suggest that little or no homology exists among the sequences of pBR322, the URA3 gene of *S. cerevisiae*, and the SV40 origin under these conditions.

Template activity of mouse DNA clones during cell extract-promoted SV40 DNA synthesis. The *in vitro* SV40 DNA replication system, consisting of the nuclear extract from HeLa cells and cytoplasmic fraction from Cos-I cells infected with SV40, promotes the initiation and subsequent elongation of DNA replication of the exogenously added SV40 DNA or cloned DNA containing the origin of SV40 DNA synthesis (4). SV40 DNA resembles a replicon in the eucaryotic genome in terms of the shape and structure of the 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. Indeed, we have already shown that one of the human interspersed repeated sequences, the *Alu* family, had a good template activity and that the initiation of DNA replication started from the *Alu* family sequences in this *in vitro* system (2).

The template activities of four DNAs, including pSVO (containing the SV40 origin [4]), pMU65, pMU111, and

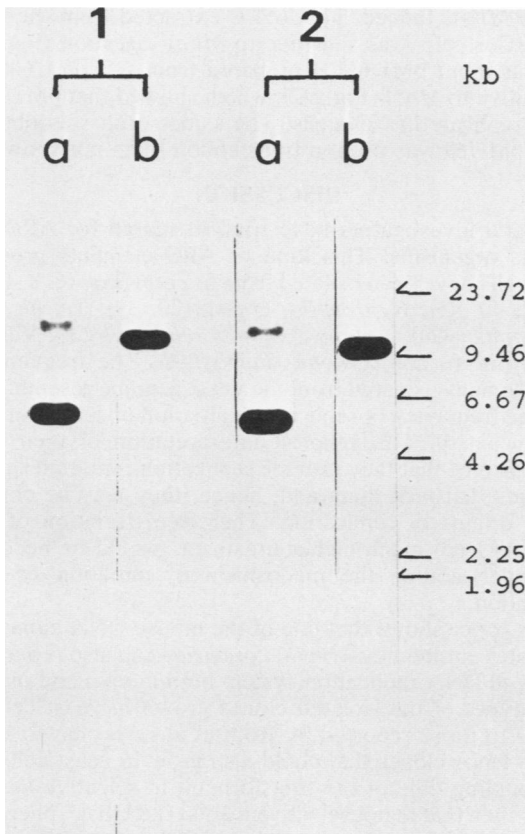


FIG. 2. Southern blot analysis of the plasmid DNA in the transformants. Total DNA extracted from yeast cells transformed with pMU65 or pMU111 was electrophoresed on a 0.8% agarose gel with (a) or without (b) treatment with *Sall*, transferred to nitrocellulose paper by the method of Southern (36), and then hybridized with nick-translated ^{32}P -labeled pBR322 DNA. Lane 1, total DNA from yeast cells transformed with pMU65; lane 2, total DNA from yeast cells transformed with untreated pMU111.

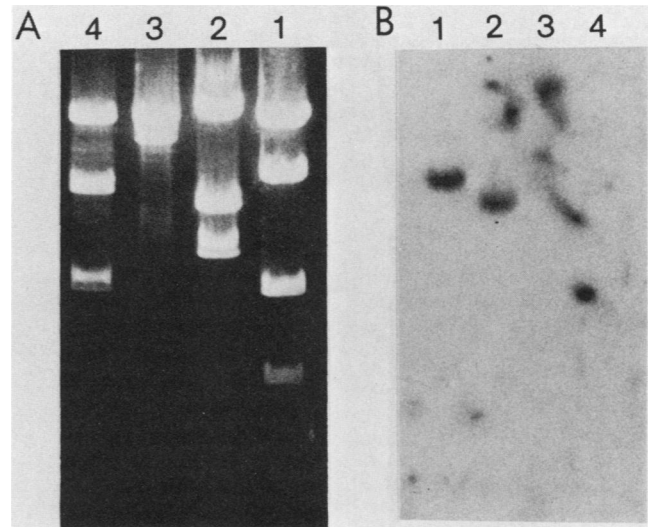


FIG. 3. Southern blot analysis of pMU65 and pMU111 DNAs. pMU65 or pMU111 DNAs (1 μg each) were cut with several restriction enzymes. DNA fragments were then separated on a 1% agarose gel, transferred to the nitrocellulose paper and hybridized with the 5' ^{32}P -labeled 13-nucleotide probe. The hybridization was carried out as described in the text by using 1.2×10^7 cpm of the probe at 60°C. (A) Ethidium bromide staining patterns of DNAs digested with several enzymes: pMU65 was cut with *Bam*HI and *Bgl*II (lane 1), pMU65 was cut with *Bgl*II and *Eco*RI (lane 2), pMU111 was cut with *Bam*HI (lane 3), and pMU111 was cut with *Bam*HI and *Hpa*I (lane 4). (B) Autoradiograms after hybridization between pMU65 or pMU111 and the ^{32}P -labeled 13-nucleotide probe. Lane numbers are the same as those in (A).

pMU115, were examined in the *in vitro* SV40 replication system, and the products were run on neutral agarose gel. The distribution patterns of labeled DNAs after autoradiography are shown in Fig. 4. pSVO was a good template and gave rise to two major bands, form I and form II, and various form I DNAs having various superhelical turns as previously described (3, 4) (Fig. 4, lane 1). Both pMU65 and pMU111 gave rise to forms II, III, and I from the top of the gel (Fig. 3, lanes 2 and 3), which were derived mainly from the semiconservative DNA replication, because we 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 as described before (2, 3) and mentioned below. On the other hand, pMU115, which was also the hybrid plasmid constructed from mouse DNA and YIp5 but could not support high-frequency transformation in yeast, gave the faint form I, II, and III DNAs (Fig. 3, lane 4), which means that pMU115 has a very weak template activity. To confirm the criterion that form I on a neutral agarose gel indicates the semiconservative DNA replication performed in this mixture, the DNAs were synthesized in a reaction mixture containing bromodeoxy-UTP in place of dTTP and analyzed by neutral CsCl equilibrium centrifugation. Approximately 97, 80, and 75% of the *in vitro* products were fully or half substituted by bromouracil in the reactions with pSVO, pMU65, and pMU111, respectively, as a template (data not shown), indicating that DNA clones tested gave a semiconservative DNA replication.

Determination of the initiation point in the reaction. DNA synthesis carried out in this mixture starts at the origin-containing region of SV40 DNA and proceeds bidirectionally (4). To assess this point in the reaction on pMU65 and

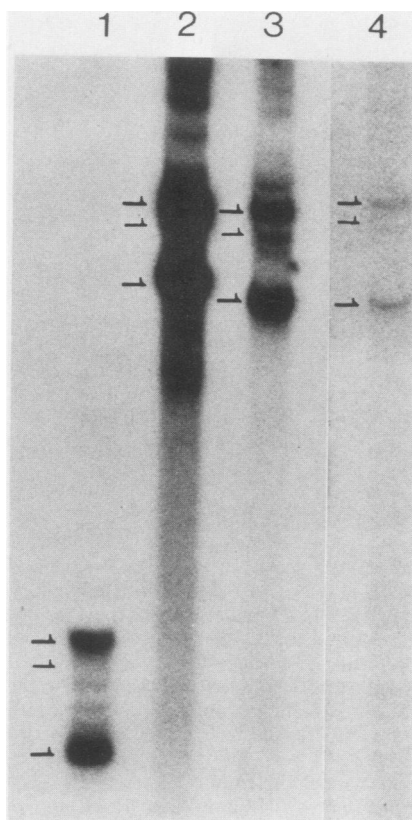


FIG. 4. DNA synthesis in vitro on various cloned DNAs. Synthesis was carried out with 0.4 μg of each DNA as a template. After 60 min at 37°C, the reaction mixture was treated with pronase and sodium dodecyl sulfate, and the DNA was precipitated with ethanol. DNA was visualized by autoradiography after agarose gel electrophoresis. Reactions were performed with pSVO (lane 1), pMU65 (lane 2), pMU111 (lane 3), and pMU115 (lane 4) as a template. Total incorporated dCMP amounts estimated by counting of 0.1-ml sample of the mixtures were 2.1, 5.3, 3.5, and 0.2 pmol/ μg of added DNA in lanes 1 through 4, respectively. The horizontal bars represent the positions of form II, III, and I DNAs from the top.

pMU111, newly synthesized DNA labeled for 5 and 60 min was extracted and digested with *EcoRI* and *BglII* (pMU65 reaction) or with *BamHI* and *HpaI* (pMU111 reaction), and the distribution of radioactivity on each fragment was examined by agarose gel electrophoresis (Table 1). Digestion of pMU65 or pMU111 with these combinations of enzymes yielded four or three fragments, respectively. The ratio of the radioactivity of each fragment was normalized to the ratio of the nucleotide numbers of each fragment. Therefore, the value 1 means that all of the sequences are equally labeled. In the first 5 min of the reaction, a very high ratio was obtained on 2.5 kb of the *EcoRI-BglII* fragment (ratio, 7.22) in the reaction on pMU65. This fragment was hybridized to the 13 ^{32}P -labeled nucleotides of the SV40 origin sequence (Fig. 3). After 60 min, the ratios of each fragment were almost 1, indicating that the label became more evenly incorporated on each fragment of pMU65. In contrast, the ratios of each fragment of pMU111 were nearly 1 after 5 or 60 min of the reaction, showing that the initiation of DNA replication starts at random throughout the DNA of pMU111. These results were parallel to those obtained in hybridization experiments in which a chemically synthesized fragment of SV40 origin was used as a probe (Fig. 3).

Replication of the plasmid DNAs in Cos cells. It is generally known that DNAs containing SV40 origin sequences can replicate autonomously in monkey Cos cells after the transfection of DNAs because Cos cells produce SV40 T antigen constitutively (15). To determine the replicating activity of our DNAs in an SV40 T-antigen-dependent system in vivo, we transfected the DNAs into Cos-I cells. After 48 h of transfection, low-molecular-weight DNA was extracted by the Hirt procedure (17), fractionated on an agarose gel, transferred to nitrocellulose, and then hybridized with nick-translated pBR322 (Fig. 5). The autoradiogram showed that pSVO, pMU65, and pMU65EB gave forms I and II (Fig. 5A, lanes 1, 2, and 3), whereas little form I or II appeared when pMU111 and pMU115 were used (Fig. 5A, lanes 4 and 5). The hybridized forms I and II were not derived from the input DNAs as shown later. The interesting point is that pMU65EB, which is a subclone of pMU65, could replicate much more frequently than pMU65 could (Fig. 5A, lanes 2 and 3), suggesting that some inhibitory sequences may exist in the region of pMU65.

To confirm that pMU65EB or pMU65 did replicate in Cos cells, the extracted DNA from the Hirt supernatant was first digested with *MboI*. Since the plasmids were propagated in *E. coli* C600 Dam⁺ bacteria, methylation of the GATC *MboI* recognition site made them completely resistant to the enzyme. Only DNA that had activity replicated in the cells would have lost the methylated adenine and become sensitive to *MboI*. Indeed, pMU65EB extracted from the transfected Cos cells was sensitive to *MboI* digestion (Fig. 5B), whereas input pMU65EB prepared from *E. coli* C600 was insensitive to *MboI* (Fig. 5C), which showed that pMU65EB could replicate in Cos cells. The same result was obtained when pMU65 was used in transfection (data not shown).

DISCUSSION

Several investigators have tried to search for ARS from various organisms. This kind of ARS elements grown in yeast cells have been isolated from *S. cerevisiae* (6, 8, 10, 18, 22, 38, 40, 45), *Neurospora crassa* (20, 39), *Tetrahymena thermophila* (24), *Chlamydomonas reinhardtii* (33; Vallet et al., in press), and *Xenopus laevis* (44). The frequency of ARS elements isolated from the yeast genome resembles the average frequency of replication initiation observed in chromosomes (10). The simplest interpretation of their effect appears to be that they provide replication-initiation sites for plasmids that lack them and, hence, they may be chromosomal origins of replication. Therefore, isolation of ARS elements from much higher organisms would be necessary for understanding the mechanism of initiation of DNA replication.

This paper shows that one of the mouse DNA clones that replicates autonomously in *S. cerevisiae* can also replicate in the SV40 DNA replication system both in vivo and in vitro. Phenotypes of the isolated clones grown in yeast cells are similar to those reported by Roth et al., who have isolated mouse DNA clones that could also grow in yeast cells (34): the doubling time of the transformant in selective media is longer than that in nonselective media, the URA⁺ phenotype of the transformant is unstable, and the original plasmids used to transform *S. cerevisiae* can be recovered from *E. coli* cells transformed with DNA extracted from the yeast transformants. In our system, the clones could replicate in Cos cells after transfection of DNA, which indicates that sequences exist that are similar to SV40 origin. These were, indeed, confirmed by hybridization experiments with a synthetic oligonucleotide probe corresponding to the SV40

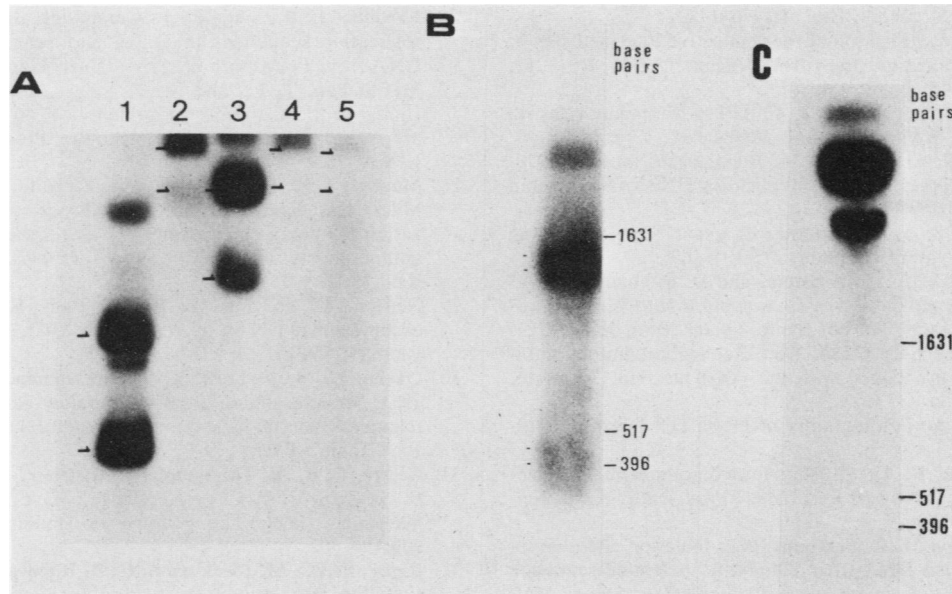


FIG. 5. Southern blot analysis of the plasmid DNAs transfected into Cos cells. (A) Samples (0.5 μ g each) of pSV0 (lane 1), pMU65 (lane 2), pMU65EB (lane 3), pMU111 (lane 4), and pMU115 (lane 5) DNAs were transfected into Cos cells by a calcium phosphate precipitation technique. At 48 h after transfection, the low-molecular-weight DNAs were extracted from the cells by the Hirt procedure (17), separated on a 1% agarose gel, transferred to the nitrocellulose filter, and hybridized with nick-translated 32 P-labeled pBR322 probe. The hybridization was carried out in the same mixtures as those described in the legend to Fig. 2 at 65°C for 16 h. The hybridized DNAs were visualized by autoradiography. Horizontal bars represent the positions of form II and I DNAs from the top. (B) pMU65EB (0.5 μ g) was transfected into Cos cells, and the low-molecular-weight DNAs were extracted from the cells as in (A). The extracted DNAs were cut with *Mbo*I, separated on a 1% agarose gel, transferred to the nitrocellulose filter, and hybridized with nick-translated 32 P-labeled pMU65EB probe. (C) pMU65EB prepared from *E. coli* C600 was cut with *Mbo*I, separated on a 1% agarose gel, transferred to the nitrocellulose filter, and hybridized with nick-translated 32 P-labeled pMU65EB probe.

origin sequences. There are several reports that describe the molecular cloning of monkey or human genomic DNA segments that hybridize to the region around the origin of SV40 (11, 27, 30). None of them, however, have been shown to replicate in the original or other higher organisms (11, 27, 30). From this point, we believe, this is the first plasmid replicating in higher eucaryotic cells.

It is not clear that the same sequences are necessary for pMU65 to show the ARS function in yeast and Cos cells. However, it is likely that ARS elements are different between the two cells because pMU111 which also has the ARS function in yeast cells can not replicate in Cos cells. We have already shown that YRp7 which contains ARS of yeast cells has no template activity in an in vitro SV40 DNA replication system (2). These results support the possibility that ARS necessary for the replication in yeast cells is different from that necessary for replication in Cos cells. Several sequencing studies of the plasmid containing the ARS function in yeast cells did not show the SV40 origin-like sequences. Another question is whether or not SV40 origin-like sequences are indeed the initiation sites of DNA replication in the original mouse or other higher eucaryotic cells. Jelinek et al. have made it apparent from nucleic acid sequence studies that there are nucleotide similarities between the *Alu* family of interspersed repeated sequences and the origin region of SV40, BK virus, and polyomavirus, suggesting that the *Alu* family might function as the origin of DNA replication in mammalian cells (23). When the template activity of one of the *Alu* family clones, BLUR8, was tested in the in vitro SV40 DNA replication system, BLUR8 had a good template activity, and the initiation of DNA replication started from the *Alu* family sequences as in the case of pMU65, which is shown here (2). However, when

the chemically synthesized 13-nucleotide sequence corresponding to the SV40 origin was cloned into the *Bam*HI site of pBR322 and was used as a template in the in vitro SV40 DNA replication system, this clone showed no template activity (data not shown), which indicates that the 13 nucleotides in the SV40 origin are the core sequence and are not sufficient for promoting DNA replication. For that, another flanking sequence present in neither the bacterial plasmid nor the yeast genome may be necessary. Furthermore, the similar functional clones which had ARS functions in the SV40 DNA replication system have also been isolated from the human lung cancer gene (H. Ariga, M. Naruto, and H. Nakano, manuscript in preparation). These were hybridized with BLUR8 and chemically synthesized SV40 origin sequences. Therefore, sequence studies of the *Eco*RI-*Bgl*III fragment of pMU65 should be done to determine the correlation between ARS of pMU65 and *Alu* family sequences.

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