Effect of Transcriptional Regulatory Sequences on Autonomous Replication of Plasmids in Transient Mammalian Systems

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Transcriptional regulatory sequences often influence the efficiency of DNA replication, directly or indirectly, in bacteria, yeast, and animal virus systems. We have tested several transcriptional regulatory sequences for affecting DNA replication, based on pUC vector, in transient systems. Autonomous replication of transfected plasmids was assayed by PCR amplification of the fragments derived from the plasmids, which had replicated in mammalian cells. By this highly efficient method of detecting replicated molecules, pUC19, but not pUC18, showed a weak replication activity in transfected cells. Nucleotide sequences around the HindIII site in pUC19 were required for replication. Monomers or dimers of the octamer transcription motif of the mouse immunoglobulin heavy chain gene, inserted in multicloning sites of pUC19, could stimulate replication, while the 4- or 6-mers did not, in contrast to the results on its transcription activity. Other transcriptional elements including AP1, HSE, and E2F also stimulated replication, but neither CRE nor Sp1 binding motif did. These results suggest that at least some of the transcriptional regulatory sequences function as modulators of DNA replication as well as of transcription.

Key words autonomous replication; transcriptional regulatory element; octamer

DNA replication is one of the most fundamental and important steps in a cell cycle, and incorrect regulation of DNA replication causes various diseases including cancer. Cellular DNA replication and transcription have been supposed, and already reported, to be mutually related, and at least some of the transcriptional regulatory sequences may be involved in DNA replication. In eukaryotic cells, chromosomal DNA replicates once in a cell cycle, at S the phase. Transcriptionally active genes generally replicate in the early S phase, while less active genes replicate in the late S phase.[1,2] Sequence-specific binding of protein factors to DNA and unwinding of the DNA helices induced by the protein factors are commonly observed for both replication and transcription. In animal viruses, transcriptional regulatory sequences such as enhancers and promoters are often found in the regions containing replication origins, and the protein factors recognizing the sequences activate, or are required for, DNA replication as well as transcription.[3–10] In mammalian cells, too, transcriptional regulatory sequences and initiation regions of DNA replication overlap in the human c-*myc*,[11–13] hamster dehydroforate reductase (DHFR),[14,15] mouse immunoglobulin heavy chain,[16,17] human heat shock 70 (hsp70),[18] and human lamin B[19] genes.

The octamer sequence, a well-characterized transcriptional element, is located in the transcriptional regulatory regions of various genes including the immunoglobulin gene,[20–23] histon H2B gene[24] and snRNA gene.[25,26] Among several different proteins reported so far to bind the octamer motif, OTF-1 (Oct-1) is ubiquitously present in a variety of cells.[27–33] Since OTF-1 is indistinguishable from NFIII, a cellular factor necessary for initiation of adenoviral DNA replication,[34–37] OTF-1 is possibly involved in cellular DNA replication as well as in transcription. We and another group have independently identified an initiation region of cellular DNA replication *in*

vivo in the enhancer of the mouse immunoglobulin heavy chain (IgH) gene, which contains the octamer sequence in the J-C intron,[16,17] and the fragment containing the octamer sequence could autonomously replicate in HeLa cells[16] and in B cell lines[17] when cloned in pUC plasmids.

In this report, we have examined the octamer motif and other transcriptional regulatory sequences for activity in stimulating DNA replication in transfected human HeLa or monkey Cos1 cells. The results suggest that at least some of the sequences affected DNA replication in addition to transcription.

MATERIALS AND METHODS

Plasmids Oligonucleotides were chemically synthesized and cloned into appropriate sites within multicloning sites of pUC19 or pUC18. The sequences of the oligonucleotides used are shown in Table 1.

Cells and Transfection Human cervical carcinoma HeLa and monkey Cos1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HeLa or Cos1 cells —50% confluent in a 6 cm dish were transfected with 5 μg test DNA and 0.1 μg internal control PCRcibi, prepared from *dam⁻ E. coli*, by the calcium phosphate precipitation technique.[38] Four hours after transfection, cells were boosted with 25% (v/v) dimethylsulfoxide for 2 min, and cultured for 2 d.

Replication Assay by PCR Procedures for replication assay by PCR were as described by Watanabe and Yamaguchi.[39] Low molecular weight DNAs were extracted from the transfected cells by the Hirt method[40] and purified by proteinase K treatment and subsequent phenol extraction. After ethanol precipitation, the DNAs were treated with RNaseA and reprecipitated with polyethyleneglycol. The DNAs were dissolved in 30 μl distilled water, and 6 μl used for assay. The DNAs were digested with DpnI at 37 °C for more than 8 h, extracted with

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Table 1. Nucleotide Sequences of the Oligonucleotides Corresponding to Various Transcriptional Elements

Name	Sequence	Cloning sites
Heptamaer/octamer	5'-agctcgagtg CTCATGA at ATGCAAAT c A sttggtcga-3'	SacI/SalI
Octamer	5'-agctcgagcccgggg T a ATTTGCAT ttctactagtcga-3'	SacI/SalI
2 × Octamer	5'-agctc (gagcccgggg T a ATTTGCAT ttctactagtc)₂ ga-3'	SacI/SalI
4 × Octamer	5'-agctc (gagcccgggg T a ATTTGCAT ttctactagtc)₄ ga-3'	SacI/SalI
6 × Octamer	5'-agctc (gagcccgggg T a ATTTGCAT ttctactagtc)₆ ga-3'	SacI/SalI
8 × Octamer	5'-agctc (gagcccgggg T a ATTTGCAT ttctactagtc)₈ ga-3'	SacI/SalI
$E^+O^{+\ a)}$	5'-ctgagcaaaac ACCACCTGGG T a ATTTGCAT ttctaaaataagtcga-3'	XhoI(EcoRI)
E^-O^+	5'-ctgagcaaaac AC ac CC g GGG T a ATTTGCAT ttctaaaataagtcga-3'	XhoI(EcoRI)
E^+O^-	5'-ctgagcaaaac ACCACCTGGG T a AT g T t CAT ttctaaaataagtcga-3'	XhoI(EcoRI)
Sp1 (SV)	5'-agctcgtaact CCGCCC gaagtcga-3'	SacI/SalI
CRE	5'-tgcaggtcgagat TGACGTCA tggtaaaaat TGACGTCA tggtc-3'	PstI/EcoRI
NFI	5'-ctagat ATTGGCT tca ATCCAAA tggatc-3'	XbaI/BamHI
AP1	5'-ctagag TTAGTCA tggatc-3'	XbaI/BamHI
HSE	5'-gat CCCTGGAATATTCCCGAC ggatc-3'	BamHI
E2F	5'-ctagagt TTTCGCGC ggatc-3'	XbaI/BamHI
SRE	5'-ct AGATGTCCTAATATGGACATCC ggatc-3'	XbaI/BamHI

Sets of complementary oligonucleotides were chemically synthesized, phosphorylated at the 5' ends, annealed and cloned into appropriate sites of pUC18 or 19 as described in Materials and Methods. Nucleotide sequences of upper strands was shown. Sp1 (SV) indicates the Sp1 sequence from the SV40 promoter. Capital letters represent consensus sequences of the elements. Cloning sites in pUC plasmids are also shown on the right. a) E, Ephrussi box; O, octamer.

phenol and chloroform, and precipitated with ethanol. Precipitated DNAs were amplified with RV and Mbo primers in a DNA thermal cycler (Perkin-Elmer). RV and Mbo primers correspond to the sequences adjacent to both ends of the multicloning sites of pUC plasmids, which are 5'-CAGGAAACAGCTATGACCATTG-3' and 5'-TTC-GATGTAACCCACTCTCGTG-3', respectively. Reaction mixture consisted of template DNA, 1 mM of each dNTP, 4 pg/ml RV primer and 4 pg/ml Mbo primer in 25 μl. The DNA was denatured at 94 °C for 1 min and annealed at 55 °C for 2 min. The extension reaction was then carried out at 72 °C for 3 min using Taq DNA polymerase. After 30 cycles of these reactions, DNAs were extracted and 1/5 was separated in a 1.2% agarose gel containing 0.5 μg/ml ethidium bromide. For further analyses according to Southern,[41] DNAs in the gel were blotted on a nitrocellulose filter and hybridized with pUC19 probe under stringent conditions.

RESULTS

Replication Activity of the pUC Plasmids in Transient Systems Vector pUC plasmids have sometimes been claimed to exhibit autonomous replicating sequence (ARS) activity in transient assay, in which DNA was harvested 2—3 d after transfection of test DNAs to cells. The plasmids pUC19 and pUC18 have the identical structure except for the multicloning sites, which have a reverse orientation. We examined pUC18 and pUC19 for ARS activity using a sensitive assay applying PCR. Conventional assay by Southern blotting has been successfully used for detecting replication of plasmids derived from animal viruses. Replication due to mammalian sequences, however, is difficult to detect consistently by similar procedures, because their copy numbers are much lower than those of the viral sequences. The PCR-mediated replication assay used in this report was originally designed for identification of the origin of cytomegaroviral replication,[39] and applied to the hsp70 gene.[18] In the method, molecules replicated de novo in cells are selec-

tively amplified more than one hundred-fold and, thereby, replication as low as 10 copies/cell can be easily detected.

pUC18 or pUC19 was co-transfected with PCRcibi, prepared from dam⁻ E. coli, to human HeLa or monkey Cos1 cells by the standard calcium phosphate method.[38] Two days after transfection, low molecular weight DNAs were extracted by the Hirt method,[40] digested with DpnI, and subjected to PCR with RV and Mbo primers (Fig. 1). pUC18 and pUC19 have 7 sites for DpnI, one of which is located between the annealing sites for RV and Mbo primers (nucleotide number 1000). Plasmids replicated in transfected cells, as well as control PCRcibi, were resistant to DpnI digestion and, hence, gave rise to 780 and 360 bp fragments, respectively. On the other hand, the residual, unreplicated pUC plasmids used for transfection, which were prepared from dam⁺ E. coli and sensitive to digestion by DpnI, could not be amplified with the primers. As shown in Fig. 3, pUC19 gave a replicated signal (Fig. 3A and 3B, lane 7), while pUC18 did not (Fig. 3A and 3B, lane 6). The signal due to pUC19 molecules replicated de novo was reproducibly detected in the PCR assay, although the replication efficiency of pUC19 was low. On the other hand, we never observed amplified fragments for pUC18. The results suggest that sequences necessary for replication exist in pUC19, but not in pUC18 which has the identical structure except for the orientation of the multicloning sites. To identify the required sequences, several deletion mutants of pUC19 were prepared and tested for replicating activity (Fig. 2). The results suggest that the sequence around the HindIII site, GCCA, and the flanking sequences of pUC19 on the 5' end of the multicloning sites are important for ARS activity.

Effect of the Octamer Motif on Replication We and another group have previously reported that an initiation site of DNA replication in vivo is located in the region overlapping the transcriptional enhancer of the mouse IgH gene[16,17] and the pUC18 clone containing this region worked transiently as an ARS in mammalian cells,[16,17] and in stable cell lines (Ogawa, Ariga and Iguchi-Ariga, manuscript in preparation). Furthermore, we have shown
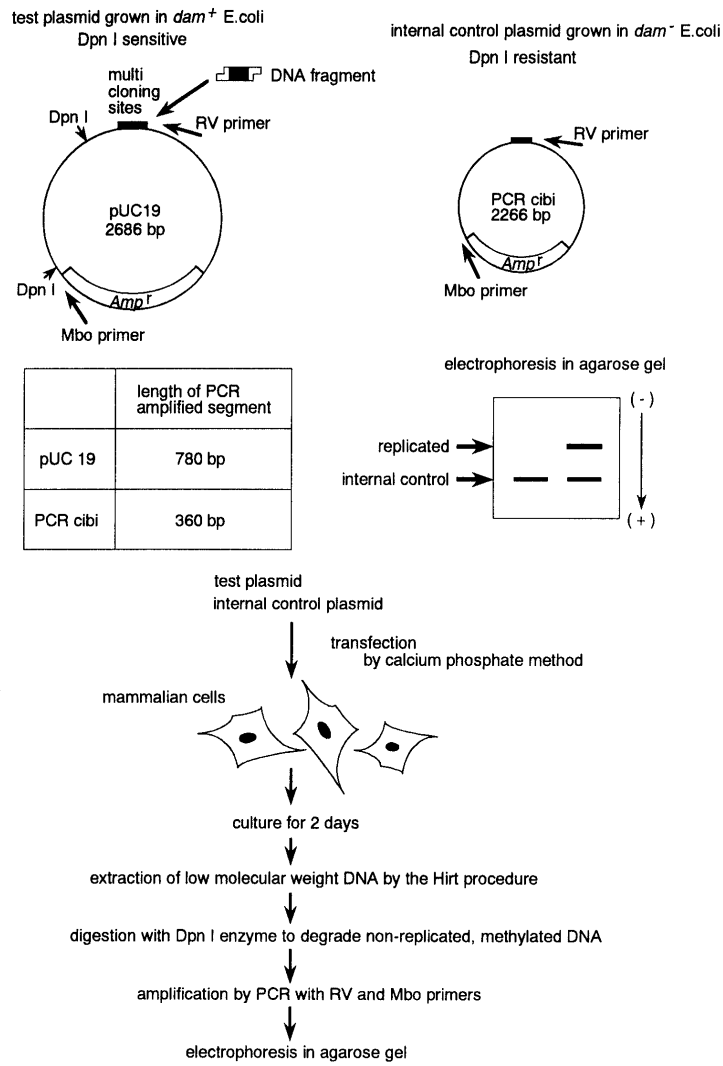
test plasmid grown in *dam⁺* E.coli
Dpn I sensitive

internal control plasmid grown in *dam⁻* E.coli
Dpn I resistant

	length of PCR amplified segment
pUC 19	780 bp
PCR cibi	360 bp

Fig. 1. Schematic Drawing of the Method of Detecting pUC Molecules Replicated in Transfected Cells

Test plasmid, prepared from *dam⁺* E. coli cells, containing the oligonucleotide in the polylinker site of pUC19 and PCRcibi, internal reference plasmid prepared from *dam⁻* E. coli cells are shown on left and right, respectively. After the DNAs recovered from transfected cells are digested with DpnI, and amplified by PCR with RV primer placed adjacent of the polylinker site and MboI primer placed outside the DpnI recognition site, pUC19 template, if replicated in the transfected mammalian cells, should give rise to the 780 bp fragment, and PCRcibi to the 360 bp one. These DNAs are detected on agarose gel.
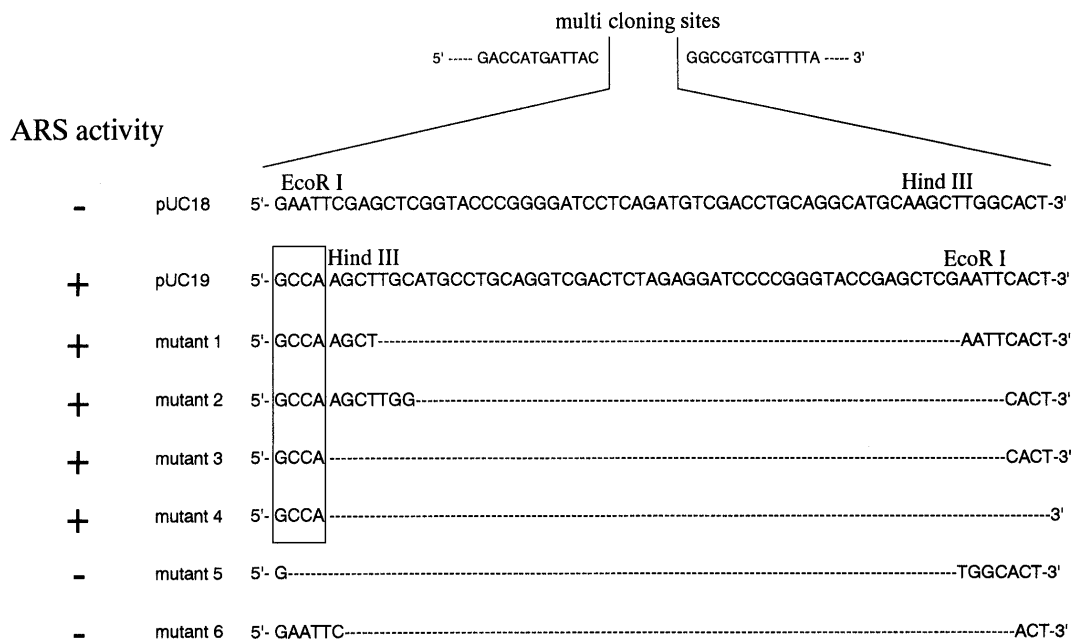

Fig. 2. Identification of the Nucleotide Sequence Necessary for Replication of pUC19 in Mammalian Cells

Nucleotide sequences in and around the polylinker site of pUC18, pUC19 and their derivatives are shown. Replication activities of the plasmids, as assayed in Fig. 1, are indicated as + or − on the left, and the sequence required for activity is indicated in the square.
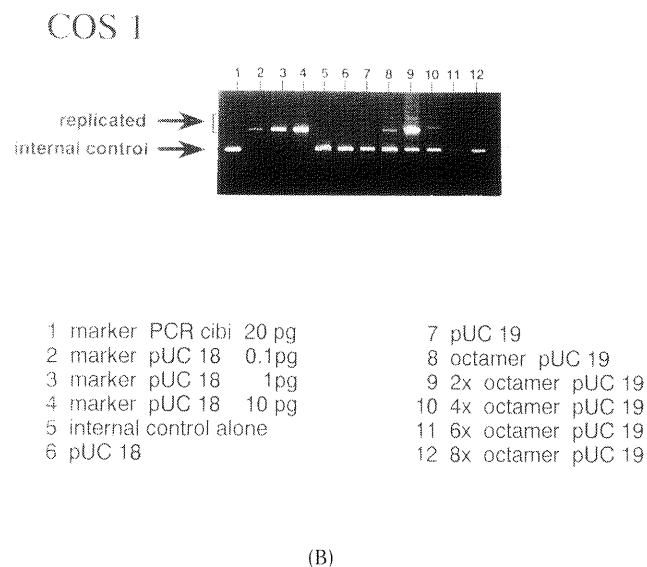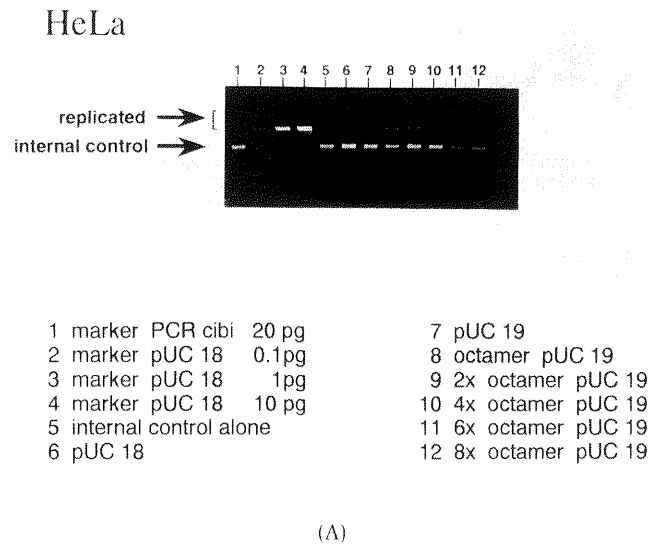
HeLa

replicated ⟶
internal control ⟶

1 marker PCR cibi 20 pg
2 marker pUC 18 0.1pg
3 marker pUC 18 1pg
4 marker pUC 18 10 pg
5 internal control alone
6 pUC 18

7 pUC 19
8 octamer pUC 19
9 2x octamer pUC 19
10 4x octamer pUC 19
11 6x octamer pUC 19
12 8x octamer pUC 19

(A)

COS 1

replicated ⟶
internal control ⟶

1 marker PCR cibi 20 pg
2 marker pUC 18 0.1pg
3 marker pUC 18 1pg
4 marker pUC 18 10 pg
5 internal control alone
6 pUC 18

7 pUC 19
8 octamer pUC 19
9 2x octamer pUC 19
10 4x octamer pUC 19
11 6x octamer pUC 19
12 8x octamer pUC 19

(B)

Fig. 3. Effect of Octamer Sequence of Replication of pUC19

pUC19 and pUC19 containing monomer , dimer, ..., of the octamer sequence were transfected to HeLa cells (A) or to Cos1 cells (B), and replication assays were carried out as in Fig. 2. Lanes 5-12 are the results from the transfected cells with plasmids shown, and lanes 1-4 are size marker controls amplified by PCR.

that the octamer (Oct) motif, a transcriptional regulatory element therein, is essential for replication.[16] The Oct motif is, thus, suggested to have an effect on replication as well as on transcription. We, therefore, examined the effect of the Oct motif on replication of pUC18 or pUC19. Multimerized Oct motifs were inserted into the polylinker site of pUC18 or pUC19, and the clones were assayed for transient replication in HeLa or Cos1 cells (Figs. 3 and 4). Compared with non-inserted pUC19, the pUC19 clones containing monomer or dimer ($\times 2$) of the Oct motif showed enhanced replication (Fig. 3, lanes 7, 8 and 9). In Cos1 cells, enhancement of replication was also observed for the clone containing the 4-mer ($\times 4$) of the motif (Fig. 3B, lane 10). The hexamer ($\times 6$) or 8-mer ($\times 8$) of the Oct motif reduced replication activity (Fig. 3, lanes 11 and 12). In the transcription system, multimerization of the Oct

motif stimulates transcription in parallel with the number of inserted Oct motifs.[22,42,43] These data suggest that the Oct motif has the ability to stimulate DNA replication, but the mode of stimulation may differ from that in transcription.

None of the pUC18 clones containing the Oct motifs replicated either in HeLa (Fig. 4) or Cos1 cells (data not shown; see Table 2). As described previously, however, pE^+O^+, another pUC18 plasmid containing the motif, could replicate in HeLa cells (Fig. 5, lane 4). pE^+O^+ possesses the Ephrussi box (E) as well as the Oct motif (O) surrounded by AT-rich sequences, corresponding to the intact IgH enhancer region[44] (Table 1). Mutation into octamer (pE^+O^-) abrogated the replication activity of the plasmid, while mutation into E box (pE^-O^+) did not (Fig. 5, lanes 5 and 6). These results suggest that in the presence

HeLa

1	octamer	pUC19
2	octamer heptamer	pUC19
3	octamer	pUC18
4	2x octamer	pUC18
5	octamer heptamer (Ssp I)	pUC18
6	pUC18	

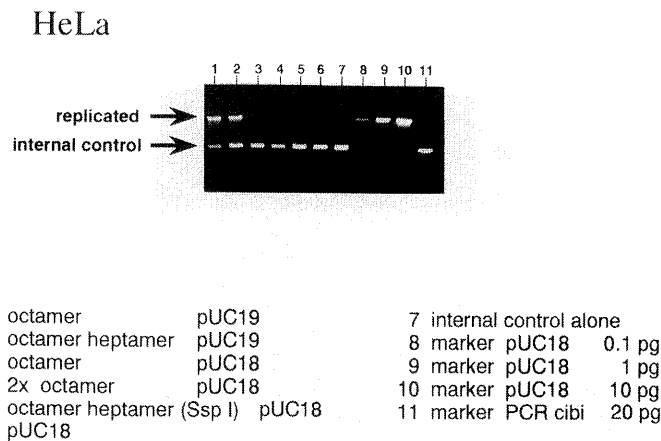| 7 | internal control alone | |
8	marker pUC18	0.1 pg
9	marker pUC18	1 pg
10	marker pUC18	10 pg
11	marker PCR cibi	20 pg

Fig. 4. Effect of Octamer Sequence of Replication of pUC18

pUC18 and pUC18 containing monomer, dimer of the octamer sequence were transfected to HeLa cells, and replication assays were carried out as in Fig. 2. Lanes 1–7 are the results from the transfected cells with plasmids shown, and lanes 8–11 are size markers amplified by PCR.

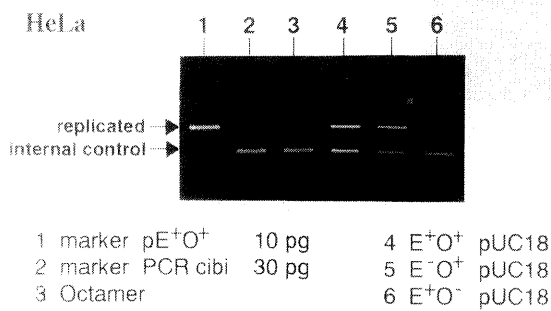| 1 | marker pE$^+$O$^+$ | 10 pg | 4 | E$^+$O$^+$ pUC18 |
| 2 | marker PCR cibi | 30 pg | 5 | E$^-$O$^+$ pUC18 |
| 3 | Octamer | | 6 | E$^+$O$^-$ pUC18 |

Fig. 5. Replication Activities of the Octamer-Ephrussi Box-Containing pUC18

Three plasmids containing intact (+) or mutated (−) octamer (O) and Ephrussi box (E), described previously[16] were tested for replication activity by PCR. Lanes 1 and 2 show the size markers amplified by PCR, and lanes 3–6 are the results for transfected cells.

Table 2. Summary of the Effects of Various Transcriptional Elements on Replication of pUC18 or 19 in Transient Systems

Vector	Transcriptional element inserted into multicloning sites	ARS activity	
		HeLa	Cos1
pUC18	None	−	−
	Octamer	−	−
	2 × Octamer	−	−
	4 × Octamer	−	−
	6 × Octamer	−	−
	8 × Octamer	−	−
	E$^+$O$^+$	+ +	n.d.[a]
	E$^-$O$^+$	+ +	n.d.
	E$^+$O$^-$	−	n.d.
	Sp1 (SV)	−	−
	CRE	−	−
	NFI	−	−
	AP1	−	−
	HSE	−	−
	E2F	−	−
	SRE	−	−
pUC19	None	+	+
	Octamer	+ +	+ +
	2 × Octamer	+ +	+ +
	4 × Octamer	+	+
	6 × Octamer	±	±
	8 × Octamer	±	±
	E$^+$O$^+$	+ +	n.d.
	E$^-$O$^+$	+ +	n.d.
	E$^+$O$^-$	−	n.d.
	Sp1 (SV)	±	±
	CRE	±	±
	NFI	+	+
	AP1	+ +	+ +
	HSE	+ +	+ +
	E2F	+ +	+ +
	SRE	±	±

a) n.d., not determined.

of neighboring AT-rich sequences, the Oct motif is responsible for the ARS activity. AT-rich sequences, which are often required for efficient replication of viral genome, may increase the potential activity of the Oct motif.

Effects of Various Transcriptional Elements on DNA Replication Various transcriptional regulatory sequences (Table 1) were inserted into polylinker sites of pUC18 or pUC19. The consequent clones were transfected to HeLa or Cos1 cells and assayed for transient replication. No clones derived from pUC18 replicated (Fig. 6). Although the results for pUC19 clones varied from experiment to experiment, the sequences of AP1, HSE and E2F reproducibly stimulated replication of pUC19 in HeLa cells (Fig. 7). CRE and the Sp1 binding sequence, on the other hand, did not show any reproducibly significant enhancement of the replication in most experiments.

HeLa

1 pmyc (H - P)
2 marker pmyc (H - P) 1pg
3 octamer heptamer pUC19
4 octamer pUC19
5 CRE pUC18
6 Sp1 pUC18
7 AP1 pUC18

8 NF1 pUC18
9 SRE pUC18
10 E2F pUC18
11 pUC18
12 internal control alone
13 marker pUC18 1pg
14 marker PCR cibi 20pg

(A)

COS 1

1 pmyc (H - P)
2 marker pmyc (H - P) 1pg
3 octamer heptamer pUC19
4 octamer pUC19
5 CRE pUC18
6 Sp1 pUC18
7 AP1 pUC18
8 NF1 pUC18

9 SRE pUC18
10 E2F pUC18
11 HSE pUC18
12 pUC18
13 internal control alone
14 marker pUC18 1pg
15 marker PCR cibi 20pg

(B)

Fig. 6. Effect of Various Transcriptional Regulatory Sequences on Replication of pUC18

Test plasmids containing various transcriptional regulatory sequences in pUC18 were transfected to HeLa (A) or Cos1 (B) cells, and the replication assays were carried out as in "Materials and Methods." Lanes 12 – 14 in A, and 13 – 15 show the size marker controls amplified by PCR. Lanes 1 – 11 in A, and 1 – 12 in B represent the results from the DNA in transfected cells.

DISCUSSION

In this manuscript, effects of transcriptional regulatory sequences on the replication of pUC plasmids were examined in a transient replication system. Replication was assayed by a PCR-mediated method. This highly sensitive method revealed that pUC19, but not pUC18, can replicate autonomously at low efficiency.

Although we reproducibly detected the replication of pUC19 in a transient system by the sensitive PCR-method described here, we did not obtain stable cell lines carrying the plasmid in the episomal state. Autonomous replication of the plasmids containing the fragment of the human c-myc gene[45,46] or the mouse IgH octamer (Ogawa, Ariga and Iguchi-Ariga, manuscript in preparation) has been observed both in transient systems and in stable trans-

formant cells. In transient systems, short sequences essential for initiation of replication may be enough for replication. Maintenance of episomal plasmids during long-term culture, however, may require additional sequences for stable replication and partition through cell divisions.

Various transcriptional elements were cloned into pUC18 or 19 and replication of the clones was tested. As summarized in Table 2, AP1,[7] HSE, and E2F[47] stimulated the replication of pUC19. These results correspond to recent reports that E2F1 and AP1 affect DNA replication: E2F1 induces quiescent cells to enter the S phase.[47] AP1[7] and other proteins of the family[10] enhance replication of polyoma virus and EB virus, respectively. The Oct motif, previously suggested to have activity on replication by our group[16] and another,[17] also enhanced
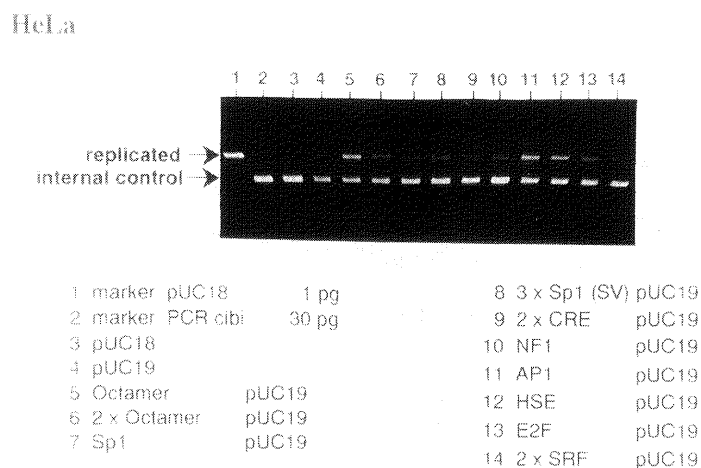
Fig. 7.   Effect of Various Transcriptional Regulatory Sequences on Replication of pUC19

Test plasmids containing various transcriptional regulatory sequences in pUC19 were transfected to HeLa cells, and the replication assays were carried out as in "Materials and Methods." Lanes 1 and 2 show the size marker controls amplified by PCR. Lanes 3—14 represent the results from the DNA in transfected cells.

pUC19 replication. CRE and the Sp1 binding sequence, on the contrary, did not significantly enhance replication of pUC19, as reported previously.[16]

Two possibilities are proposed for enhancement of DNA replication by these transcriptional elements. Binding of specific factors to the transcriptional elements enhances the weak replication initiated at the pUC19 sequence. All the constructs preserve the sequences around the 5′-end of multicloning sites, identified here as required for replication of pUC19. The factor binding may bring about structural changes in DNA, namely opening or unwinding of double strands and/or bending, which would benefit replication. Alternatively, the transcriptional elements themselves may contain the sequences required for initiation of DNA replication, and the factors recognizing them may, hence, work as initiator proteins. None of the elements, including those with ARS activity in pUC19, replicated when cloned in pUC18. The elements alone are, thus, suggested to be insufficient for replication. Only the Oct motif followed by the adjacent AT-rich sequences as in the intact IgH gene (see Table 1 for E^+O^+ and E^-O^+) showed replicating activity in pUC18 as well as in pUC19. Appropriate flanking sequences are, hence, suggested to bring the replicating activity of potential ARSs. Other transcriptional elements might also serve as a replication origin in the presence of different sequences. The octamer motif by itself showed replicating activity in the monomer, dimer, and tetramer forms in pUC19, but the same motif no longer showed any activity in further multimerized forms, in contrast to its transcriptional activity. The results also suggest that not only enhancing elements but also the whole structure, including surrounding sequences, determine the replication efficiency.

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