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A Replication-Enhancing Element with Transcriptional Silencer Activity in Autonomously Replicating Human Chromosomal Fragments

Chikashi Obuse,* Yukiko Okuno,† Tuneko Okazaki, and Hisao Masukata†‡

Department of Molecular Biology, School of Science, Nagoya University, Nagoya 464–01, Japan

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We have identified specific nucleotide sequences involved in autonomous replication of human chromosomal fragments in human cells. Nested deletion analysis of a 10.2-kb long human chromosomal fragment showed that replication efficiency of the fragment was reduced to about 50% by loss of a short specific segment. Deletions outside the segment reduced the replication efficiency depending on their lengths. By introducing linker substitutions, we found that the distinct segment required for the efficient replication consisted of an 18-bp sequence, named REE1 (Replication Enhancing Element 1). Single or tandem copies of REE1 alone had no significant replication activity, but they stimulated replication of human chromosomal DNA fragments. We found, in addition, that the REE1 sequence inserted at a site 2.7 kb upstream of the SV40 early promoter caused repression of transcription from the promoter, suggesting that REE1 had a transcriptional silencer activity. Introduction of linker substitutions into the REE1 indicated that the nucleotide sequences required for the repression of transcription were the same as those for enhancement of replication. Thus, REE1 is responsible for both enhancement of replication and repression of transcription.

INTRODUCTION

Initiation of chromosomal replication in eukaryotic cells is tightly regulated in the cell division cycle. To understand the mechanisms of initiation of chromosomal DNA replication and its regulation, identification of specific nucleotide sequences participating in the initiation of replication is considered to be necessary. In the budding yeast, *Saccharomyces cerevisiae*, short distinct chromosomal fragments that can replicate autonomously have been identified (Hsiao and Carbon 1979; Struhl *et al.*, 1979). Some of the autonomously replicating sequences (ARSs) have been shown to function as chromosomal replication origins (Huberman *et al.*, 1988; Linskens and Huberman, 1988). All of the yeast ARSs contain a match to an 11-bp (bp) ARS consensus sequence that is essential for ARS function (Broach *et al.*, 1983; Van Houten and Newlon, 1990). A detailed analysis of ARS1, which has proven to be a chromosomal replication origin, revealed that in addition to an essential A element containing the ARS consensus sequence, three additional elements, B1, B2, and B3, are required for efficient ARS function (Marahrens and Stillman, 1992). Recent studies showed that the element A is the site for binding of the origin recognition complex consisting of six protein components (Bell and Stillman, 1992; Bell *et al.*, 1993). Genetic evidences showed that at least some of these components are involved in transcriptional silencing at the mating type controlling locus (Foss *et al.*, 1993; Li and Herskowitz, 1993; Micklem *et al.*, 1993). In addition, ABF1, which binds to the element B3, is known to function in transcriptional regulation of many yeast genes (Shore *et al.*, 1987; Buchman *et al.*, 1988; Marahrens and Stillman, 1992). These findings show that specific nucleotide sequences required for the function of the yeast replication origin are recognized by protein factors that are also involved in transcriptional regulation.

* Present address: Nara Institutes of Science and Technology, 8916–5 Takayama, Ikoma, Nara 630–01, Japan.
† Present address: Faculty of Science, Osaka University, Machikaneyama-chou, Toyonaka, Osaka 560, Japan.
‡ Corresponding author.

In contrast to the budding yeast replication origin consisting of distinct sequence elements, nucleotide sequences involved in the initiation of the chromosomal replication in higher eukaryotic cells have not been discovered. It has been suggested that chromosomal replication in animal cells is initiated from preferred loci (reviewed in DePamphilis, 1993). The most extensive analysis of mammalian DNA replication has been carried out for the dihydrofolate reductase (*DHFR*) gene locus of Chinese hamster ovary cells. The results by using a variety of methods showed that the replication is initiated from a specific region as small as a 450-bp segment in the highest resolution (Anachkova and Hamlin, 1989; Handeli *et al.*, 1989; Leu and Hamlin, 1989; Burhans *et al.*, 1990; Vassilev *et al.*, 1990). However, two-dimensional gel electrophoresis analysis of the initiation sites in the locus showed that the sites are distributed within a 55-kilobase pair (kb) region containing the segment defined by other methods (Vaughn *et al.*, 1990; Dijkwel and Hamlin, 1992). Models to accommodate these different results have been proposed (Linskens and Huberman, 1990; DePamphilis, 1993). At present, no distinct sequence elements responsible for the initiation either from the narrow region or from a broad initiation zone in the *DHFR* locus have been identified. On the other hand, the presence of specific segments responsible for the initiation of replication in animal cells has been suggested for two chromosomal loci: the *Drosophila* chorion gene region (Orr-Weaver, 1991) and the human β-globin region (Kitsberg *et al.*, 1993). Extensive genetic analyses of the elements responsible for the amplification of the chorion gene cluster during oogenesis of *Drosophila* identified the amplification control element and the amplification-enhancing regions (Orr-Weaver and Spradling, 1986; Delidakis and Kafatos, 1989). Although the amplification control element is sufficient to support developmentally regulated amplification, the actual replication is initiated from multiple sites within an 8-kb region containing the amplification-enhancing regions, which stimulate the amplification (Heck and Spradling, 1990; Carminati *et al.*, 1992). In human cells, the initiation of replication from a 2-kb segment upstream of the β-globin gene was eliminated by an 8-kb natural deletion, although the specific sequence element responsible for initiation of replication remains to be identified (Kitsberg *et al.*, 1993).

A straight forward approach for identifying specific sequences required for initiation of replication, such as isolation of ARSs, has not been successful in animal cells. Replication activities of short chromosomal fragments, which had been reported to replicate autonomously in mammalian cells (Frappier and Zannis-Hadjopoulos, 1987; Iguchi-Ariga *et al.*, 1988), have not been confirmed (Burhans *et al.*, 1990; Caddle and Calos, 1992; Masukata *et al.*, 1993). Moreover, a several kilobase pair fragment containing the defined initiation sites downstream of the hamster *DHFR* gene failed to replicate autonomously in various cell lines (Burhans *et al.*, 1990; Caddle and Calos, 1992). Although a longer fragment (13 kb) including the same region replicated significantly, its replication activity was not greater than those of randomly cloned human chromosomal fragments (Caddle and Calos, 1992). A series of reports from Calos's group showed that replication of randomly cloned human fragments in human cells required substantial length of the fragments without any apparent dependency on nucleotide sequences (Krysan *et al.*, 1989, 1993; Heinzel *et al.*, 1991). However, our analysis for autonomous replication of human chromosomal fragments in human cells showed that certain fragments replicated several times more efficiently than other fragments of a similar length, suggesting the presence of specific sequences required for replication of human chromosomal fragments (Masukata *et al.*, 1993).

In the present study, we focused on identification of specific nucleotide sequences involved in autonomous replication of human chromosomal fragments and showed that a distinct 18-bp DNA element is responsible for the efficient replication. The element alone is not sufficient for replication, but can enhance replication of human chromosomal fragments. We found that the element has a transcriptional silencer activity. The results suggest that the element is involved both in regulation of transcription and in initiation of replication of human chromosomal fragments.

MATERIALS AND METHODS

Bacterial Strain

To prepare plasmid DNA, transformed *Escherichia coli* DH5a cells (Dower *et al.*, 1988) were grown in LB broth (10 g bactotrypton, 5 g yeast extract, and 10 g NaCl in 1 liter of distilled water at pH 7.2) supplemented with 30 μg/ml ampicillin and 60 μg/ml methicillin (Masukata and Tomizawa, 1986). Bacterial cells were cultured at 30°C instead of 37°C to reduce rearrangement of the plasmids. Plasmid DNA extracted from the bacteria by the Triton-Lysozyme method (Clewell and Helinski, 1972) was purified by two successive centrifugations in a CsCl solution containing ethidium bromide, followed by phenol extraction and ethanol precipitation.

Cell Culture

The cell line 293S is a suspension-adapted derivative of a human embryonic kidney cell line (293) transformed by adenovirus type 5 DNA (Stillman and Gluzman, 1985). The cells were cultured at 37°C under 5% CO_2 in DMEM supplemented with 10% fetal bovine serum.

Oligonucleotides

The oligonucleotides used for construction of plasmids are shown below.
NS1, 5′-GCGGCCGCCCTGCAGG-3′;
NS2, 5′-CCTGCAGGGCGGCCGC-3′;
TL1, 5′-AATTGGCCACCCTGG$(CCCTAA)_{10}$C-3′;

TL2, 5′-TGCAG(TTAGGG)$_{10}$CCAGGGTGGCC-3′;
TL3, 5′-TGCAGCATATGTGCACGCGTATCGATATCATGAATTC-GAAGCTTAAGATCTGCAGGATCCATGGGCCCGGGCT-AGCGGCCGCATG-3′;
TL4,5′-CGGCCGCTAGCCCGGGCCCATGGATCCTGCAGATCTT-AAGCTTCGAATTCATGATATCGATACGCGTGCACATA-TGC-3′;
TL5, 5′-C(TTAGGG)$_{10}$CCAGGGTGGCC-3′;
TL6, 5′-AGCTGGCCACCCTGG(CCCTAA)$_{10}$GCATG-3′;
RE1F, 5′-CGAAACACATGTTGCTGTCCATGGTCCTAAATAA-3′;
RE1R, 5′-TCGTTATTTAGGACCATGGACAGCAACATGTGTT-3′;
RX1F, 5′-CGAAACACATGTTGCTGTCCTCGAGCCTAAATAA-3′;
RX1R, 5′-TCGTTATTTAGG CTCGAGGACAGCAACATGTGTT-3′;

The oligonucleotides used for the polymerase chain reaction (PCR) are shown below. The names of the oligonucleotides represent the positions in the pW1–1 sequence corresponding to the left most bases of the *Xho*I substitutions (underlined).

682F/5′-TTGACAGGTGGACAAAGG-3′
1101R/5′-CTTAGAACTGAATATGCA-3′
X883F, 5′-ACA CTCGAGATATAGCCTAACCTAA-3′;
X883R, 5′-TAT CTCGAGTGTCAGGGAGCATATT-3′;
X903F, 5′-CCT CTCGAGCTGCGAGAGAGGTGG-3′;
X903R, 5′-CAG CTCGAGAGGTTAGGCTATATCC-3′;
X921F, 5′-GTG CTCGAGCATGTTGCTGTCCATG-3′;
X921R, 5′-ATG CTCGAGCACCTCTCGCAGAAAC-3′;
X922F, 5′-TGG CTCGAGATGTTGCTGTCCATGG-3′;
X922R, 5′-CAT CTCGAGCCACCTCTCGCAGAAA-3′;
X924F, 5′-GAA CTCGAGGTTGCTGTCCATGGTC-3′;
X924R, 5′-AAC CTCGAGTTCCACCTCTCGCAGA-3′;
X926F, 5′-AAC CTCGAGTGCTGTCCATGGTCCT-3′;
X926R, 5′-GCA CTCGAGGTTTCCACCTCTCGCA-3′;
X927F, 5′-ACA CTCGAGGCTGTCCATGGTCCTA-3′;
X927R, 5′-AGC CTCGAGTGTTTCCACCTCTCGC-3′;
X930F, 5′-CAT CTCGAGGTCCATGGTCCTAAAT-3′;
X930R, 5′-GAC CTCGAGATGTGTTTCCACCTCT-3′;
X933F, 5′-GTT CTCGAGCATGGTCCTAAATAAT-3′;
X933R, 5′-ATG CTCGAGAACATGTGTTTCCACC-3′;
X939F, 5′-GTC CTCGAGCCTAAATAATCACTGA-3′;
X939R, 5′-AGG CTCGAGGACAGCAACATGTGTT-3′;
X944F, 5′-TGG CTCGAGATAATCACTGAGCACA-3′;
X944R, 5′-TAT CTCGAGCCATGGACAGCAACAT-3′;
X947F, 5′-TCC CTCGAGATCACTGAGCACAGTT-3′;
X947R, 5′-GAT CTCGAGGGACCATGGACAGCAA-3′;
X948F, 5′-CCT CTCGAGTCACTGAGCACAGTTT-3′;
X948R, 5′-TGA CTCGAGAGGACCATGGACAGCA-3′;
X949F, 5′-CTA CTCGAGCACTGAGCACAGTTTTTG-3′;
X949R, 5′-GTG CTCGAGTAGGACCATGGACAGC-3′;
X950F, 5′-TAA CTCGAGACTGAGCACAGTTTTTG-3′;
X950R, 5′-AGT CTCGAGTTAGGACCATGGACAG-3′;
X956F, 5′-ATC CTCGAGCACAGTTTTTGTTTGT-3′;
X956R, 5′-GTG CTCGAGGATTATTTAGGACCAT-3′;
X965F, 5′-CAC CTCGAGTGTTTGTTTGTTTTCT-3′;
X965R, 5′-ACA CTCGAGGTGCTCAGTGATTATT-3′;
X973F, 5′-TTG CTCGAGTGTTTTCTCCTAGCAC-3′;
X973R, 5′-ACA CTCGAGCAAAAACTGTGCTCAG-3′;
X981F, 5′-TTG CTCGAGCCTAGCACTGCCTCAG-3′;
X981R, 5′-AGG CTCGAGCAAACAAACAAAAACT-3′;
X990F, 5′-CCT CTCGAGGCCTCAGCACATCCCA-3′;
X990R, 5′-GGC CTCGAGAGGAGAAAACAAACAA-3′;
X1001F, 5′-CTC CTCGAGTCCCAACTGAGCATCA-3′;
X1001R, 5′-GGA CTCGAGGAGGCAGTGCTAGGAG-3′;
X1027F, 5′-GTG CTCGAGAGAGATGGAGGTAAAA-3′;
X1027R, 5′-TCT CTCGAGCACCTGATGCTCAGTT-3′.

Construction of Nested Deletion Derivatives of pW1–1

Plasmid pW1–1 carries a 10.2-kb *Hin*dIII fragment of human chromosome at the *Hin*dIII site of pUC119 (Masukata *et al.*, 1993). The end of the insert proximal to an internal *Eco*RI site was arbitrarily called the left end and assigned the position 1 for the nucleotide sequences. A cloning vector pUCTL1 was made by replacing the *Hin*dIII–*Eco*RI cloning-site segment of pUC119 with six synthetic oligonucleotides, TL1–TL6, containing multicloning sites. The 10.2-kb *Hin*dIII fragment of pW1–1, after end-filling reaction with the *E. coli* DNA polymerase I large fragment (Klenow fragment), was inserted at the unique *Eco*RV site of pUCTL1. The resulting pWL1 and pWR1, respectively, contain the left and right ends of the insert proximal to the *Hin*dIII site of the vector. For construction of unidirectional nested deletions from either end of the insert, a double-stranded Nested Deletion Kit (Pharmacia, Piscataway, NJ) was used as recommended by the manufacturer. Briefly, 5 μg of pWL1 (series L) or pWR1 (series R) were digested with *Not*I, followed by end-filling with the Klenow fragment in the presence of 4dNTPαS mixture and then digested with *Hin*dIII at the site proximal to the insert. The linearized DNA was digested with *E. coli* exonuclease III and successively with nuclease S1. The DNA circularized with T4 DNA ligase was used for transformation of *E. coli* DH5α by electroporation. The nucleotide sequences of the retaining DNA were determined using an ALF DNA sequencer (Pharmacia). The name of each deletion derivative was designated by the position of the first natural nucleotide sequence retained.

pWNS1402 carrying the unique *Not*I and *Sse*8387I sites, in this order from the left, was made, first by eliminating the *Not*I site in the multicloning sites of pWL1 by end-filling reaction, and then by inserting synthetic oligonucleotides NS1 and NS2 into the *Eco*RI site (position 1402). For the construction of deletion series M, pWNS1402 was linearized with *Not*I and *Sse*8387I and used for the exonuclease III reaction as described above.

Construction of Linker Substitution Derivatives of pW1–1

The *Bgl*II site (position 706) of pWL1 was altered to the *Not*I site by partial digestion with *Bgl*II followed by end-filling reaction and ligation with a *Not*I linker (dpGCGGCCGC). Then the *Nhe*I site (position 1075) of the resulting plasmid was similarly changed to the *Sse*8387I site by *Sse*8387I-linker (dpCCTGCAGG) insertion. The resulting pWN706S1075 was used as the template for PCR with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). The PCR products made on pWN706S1075 using a set of the outer primer 682F and a mutagenic R-primer were digested with *Not*I and *Xho*I (PCR-N fragments). Similarly, PCR products using the outer primer 1111R and a mutagenic F-primer were digested with *Sse*8387I and *Xho*I (PCR-S fragments). A pair of PCR-N and -S fragments were inserted in place of the *Not*I–*Sse*8387I segment of pWN706S1075, resulting in substitution of 6-bp natural sequences with an *Xho*I site.

Construction of Other Plasmids

To delete the segment from positions 1 to 1402, pWNS1402 was digested by *Sma*I and *Not*I, and self-ligated after end-filling reaction with the Klenow fragment. The *Sse*8387I site of the resulting plasmid was substituted with an *Xho*I site by digestion of overhanging ends with nuclease S1 and insertion of an *Xho*I linker, resulting in pWΔ1402. The PCR products made on pWN706S1075 using primers 921F and 950R were digested by *Xho*I and inserted at the *Xho*I site of pWΔ1402 to make pWΔ1402RE1. To make tandem repeats of the RE1 fragment, oligonucleotides RE1F and RE1R were phosphorylated by T4 polynucleotide kinase, annealed, and the ligated DNA was separated by 2% agarose gel electrophoresis after end-filling with the Klenow fragment. The band corresponding to the 10-mer fragment was excised and inserted at the *Sma*I site of a pUC119

derivative, which had an additional *Sal*I site in place of the *Eco*RI site. The resulting pUCRE1$_{10}$ has 10 tandem copies of RE1 segment, which will be excised by *Sal*I digestion. Similarly, pUCRX1$_{10}$ was made by insertion of 10 tandem copies of oligonucleotides RX1F and RX1R carrying the *Xho*I substitution. Insertion of the *Sal*I fragment of pUCRE1$_{10}$, in the natural and opposite orientations, at the unique *Xho*I site of pWX933 resulted in pWX933RE1$_{10A}$ and pWX933RE1$_{10B}$, respectively. Similarly, pWX933RX1$_{10A}$ and pWX933RX1$_{10B}$ were made by insertion of the *Sal*I fragment of pUCRX1$_{10}$. To insert 10 copies of the RE1 fragment at other places in pWX933, the *Xho*I site at position 933 was eliminated by end-filling reaction and an *Xho*I linker was inserted at either *Sma*I site in the multicloning sites or the *Sna*BI site at position 9999. Insertion of the *Sal*I fragment of pUCRE1$_{10}$ at the created *Xho*I site resulted in pWX0RE1$_{10}$ or pWX9999-RE1$_{10}$, respectively. Insertion of the *Sal*I fragment of pUCRE1$_{10}$ at the multicloning sites in pUC119-derivatives carrying various human chromosomal fragments, p4A5, p1–205, p1–213, and p5–205 (Masukata *et al.*, 1993), resulted in p4A5RE1$_{10}$, p1–205RE1$_{10}$, p1–213RE1$_{10}$, and p5–205RE1$_{10}$, respectively.

The luciferase reporter plasmid, pGLPE was made by insertion of the *Hin*dIII–*Pvu*II fragment of pSV2neo (from positions 5178 to 270 of the SV40 sequence) containing the SV40 early promoter and enhancer into the proximal *Hin*dIII–*Sma*I sites upstream of the luciferase gene in pGL2-Basic (Promega, Madison, WI). To construct pGLP lacking the enhancer segment, the *Hin*dIII–*Sph*I fragment (from position 5178 to 118 of the SV40) was inserted at the *Hin*dIII–*Sph*I sites of pUC119 and then the *Hin*dIII–*Sal*I fragment of the resulting plasmid was inserted into the *Hin*dIII–*Xho*I sites of pGL2-Basic. The PCR products made on pW1–1 using X903F and X965R primers were digested by *Xho*I and inserted at the unique *Sal*I site 2.7 kb upstream of the SV40 early promoter in pGLPE and pGLP, resulting in pGLPE-RE1$_1$ and pGLP-RE1$_1$, respectively. The corresponding PCR product made on pWX933K was used to construct pGLPE-RX1$_1$ and pGLP-RX1$_1$. Insertion of the the *Sal*I fragment of pUCRE1$_{10}$ into the *Sal*I site of pGLPE in either orientation made pGLPE-RE1$_{10A}$ and pGLPE-RE1$_{10B}$. Insertion of *Sal*I fragment of pUCRX1$_{10}$ at the same site made pGLPE-RX1$_{10A}$ and pGLPE-RX1$_{10B}$. Derivatives of pGLP were similarly constructed. Plasmid pGLPE-W was made by insertion of the *Not*I–*Sse*8387I fragment (from positions 706 to 1075) of pWN706S1075 at the *Not*I–*Sse*8387I sites in pGLNS, which had been constructed by insertion of oligonucleotides NS1 and NS2 at the filled-in *Bam*HI site 2.7 kb upstream of the SV40 promoter of pGLPE. The corresponding fragments of pWX926, pWX930, pWX933, pWX939, pWX944, pWX947, and pWX950 were used to make derivatives of pGLPE carrying the *Xho*I substitutions.

DNA Replication Assay in Human Cells

Methods to examine autonomous replication in human cells were as described previously (Masukata *et al.*, 1993).

Luciferase Assay

Transfection of human 293S cells was carried out as described previously (Masukata *et al.*, 1993), except for 0.01 μg of a test plasmid, 0.1 μg of pMiwZ (Suemori *et al.*, 1990) containing the β-galactosidase gene for normalization of transfection efficiency, and 3 μg of pUC119 as carrier DNA were used. At 36 h after transfection, the cells were lysed in 0.25 ml of lysing solution containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra acetic acid, 10% glycerol, and 1% Triton X-100, and centrifuged at 15000 rpm for 10 s. For the assay, 20 μl of the supernatant was mixed with 100 μl of reaction buffer containing 20 mM tricine (pH 7.8), 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP, and the luciferase activity was measured by Lumat LB9501 luminometer (EG & G Berthold) for 20 s at room temperature. To normalize transfection efficiency, β-galactosidase activity in the cell extract was measured as described (Herbomel *et al.*, 1984).

Nucleotide Sequence Accession Number

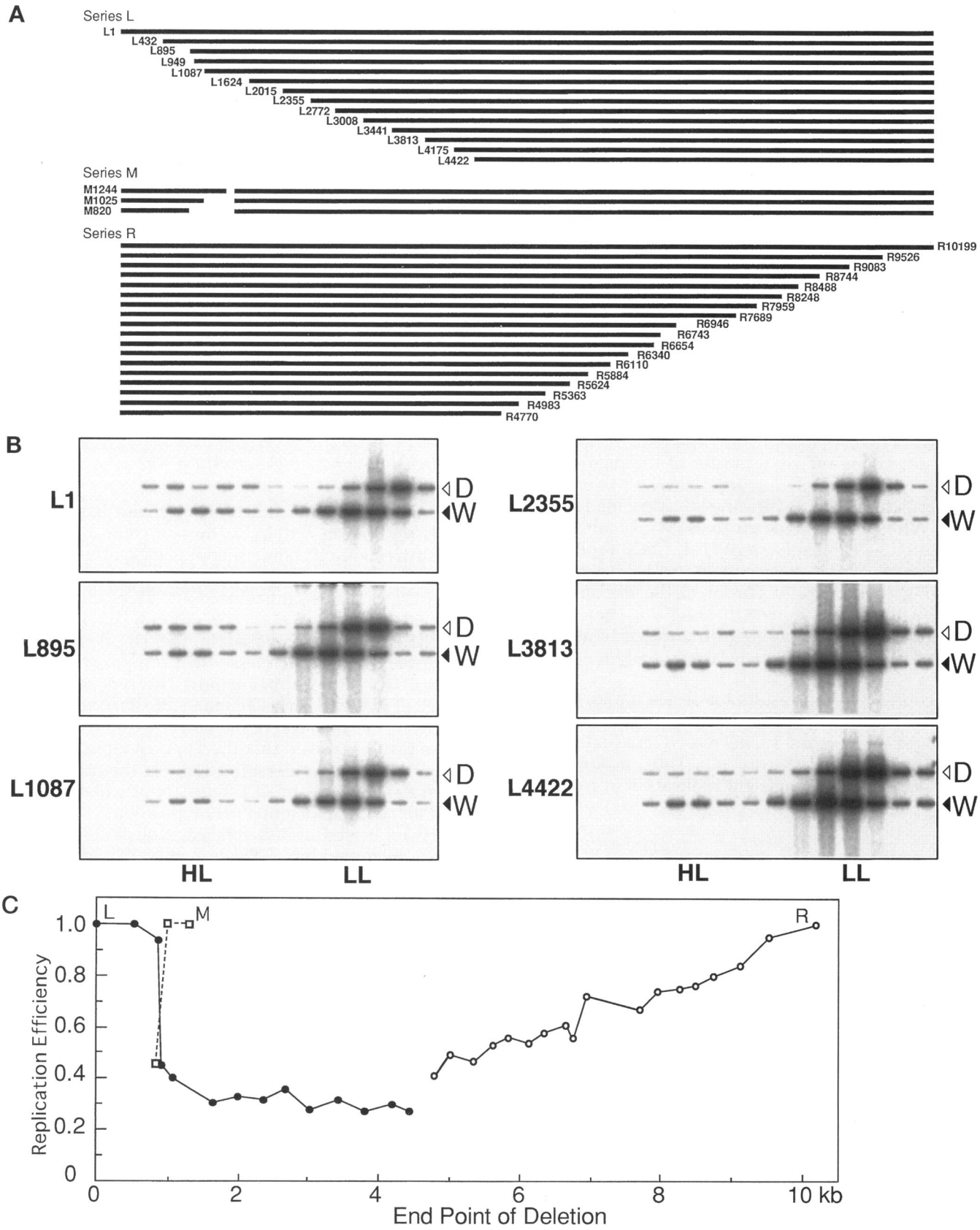
The DDBJ/GenBank/EMBL accession number for W1–1 is D50561.

RESULTS

Nested Deletion Analysis of the Regions Responsible for Replication of a Human Chromosomal Fragment

We have shown previously that a 10.2-kb fragment of human chromosome W1–1, cloned into pUC119, replicated several fold more efficiently than randomly cloned fragments in the transfected human 293S cells (Masukata *et al.*, 1993). To delimit the region responsible for replication of the W1–1 fragment, a series of deletion derivatives lacking various segments from the left end of the fragment (series L) was constructed (Figure 1A). The human 293S cells were transfected with a deletion derivative together with the internal control pW1–1 carrying the intact fragment, and were labeled with bromodeoxyuridine for 24 h from 2 days after the transfection. The extrachromosomal DNA (Hirt extract) prepared was fractionated by CsCl equilibrium density gradient centrifugation after linearization with appropriate restriction enzymes and analyzed by Southern hybridization with ^{32}P-labeled pUC119 DNA. As shown in Figure 1B, approximately 15% of pW1–1 was found in the heavy-light (HL)

Figure 1 (facing page). Effect of deletions on autonomous replication of pW1–1. (A) Derivatives of pW1–1 with nested unidirectional deletions were constructed as described in MATERIALS AND METHODS. Series L (top), M (middle), and R (bottom) contain deletions made from the left end, from position 1402, and from the right end of the insert, respectively. Regions retaining the natural sequence in the deletion derivatives are shown by solid lines. The names of deletion derivatives designate the positions of the first natural sequence retained. (B) Human 293S cells were transfected with a deletion derivative and pW1–1 as an internal control. After 2 days, the cells were labeled with bromodeoxyuridine for 24 h, which corresponds to about one cell generation, and the extrachromosomal DNA was digested with *Hin*dIII and *Bam*HI, and fractionated by CsCl equilibrium density gradient centrifugation. The DNA in each fraction was separated by 0.8% agarose gel electrophoresis, blotted, and hybridized with ^{32}P-labeled pUC119 probe. The names of deletion derivatives are shown on the left of the respective panels. The 3.2-kb vector fragment derived from the control plasmid is indicated as (W) on the right of each panel. The vector containing fragments derived from deletion derivatives, shown by (D), are larger than that of the control plasmid because of elimination of the *Hin*dIII site in the multicloning site. The positions of the light-light (LL) and heavy-light (HL) densities are shown below the panel. (C) Replication activities of deletion derivatives in the series L (●), M (□), and R (○) relative to the parental plasmid are shown. The radioactivity of each band shown in panel B was measured by Fuji Image Analyzer BAS1000 and the replication efficiency was calculated as the ratio of one-half of radioactivity of the HL-DNA to the total radioactivity. Values presented are averages of the relative replication efficiencies in at least three independent experiments.


A
Series L
L1
L432
L895
L949
L1087
L1624
L2015
L2355
L2772
L3008
L3441
L3813
L4175
L4422
Series M
M1244
M1025
M820
Series R
R10199
R9526
R9083
R8744
R8488
R8248
R7959
R7689
R6946
R6743
R6654
R6340
R6110
R5884
R5624
R5363
R4983
R4770
B
L1
L2355
L895
L3813
L1087
L4422
D
W
HL
LL
C
Replication Efficiency
1.0
0.8
0.6
0.4
0.2
0
L
M
R
0
2
4
6
8
10 kb
End Point of Deletion

density fraction (the lower bands in Figure 1B). Fractions of L1 and L895 DNA recovered in the HL density (the upper bands in Figures 1B) were almost the same as the control plasmid. In contrast, the fractions of L1087, L2355, L3813, and L4422 with longer deletions recovered in the HL density were about one-half of the control plasmid.

The efficiency of replication of each plasmid was expressed as the ratio of one-half of the HL radioactivity to the total radioactivity, and the replication efficiencies relative to the value of the internal control were calculated. As shown in Figure 1C, in contrast to the deletion derivatives L432 and L895 that replicated as efficiently as pW1–1, L949 with an additional 50-bp deletion replicated at about 40% of the efficiency of pW1–1. Replication efficiencies were reduced only slightly from the level of L949 by longer deletions. These results showed that a discrete region near the left end of the insert was required for efficient replication of pW1–1. The left border of the region was located between positions 895 and 948.

To determine the right border of the region, derivatives with internal deletions (series M) extending from position 1402 toward the left were made (Figure 1A). While deletion derivatives M1244 and M1025 replicated as efficiently as pW1–1, M820 replicated at about 50% of the efficiency of pW1–1 (Figure 1C), showing that the right border of the region was located between positions 821 and 1025. Therefore, we conclude that the region required for efficient replication of pW1–1 is located between positions 895 and 1025.

In contrast to the results for series L and M, replication efficiencies of derivatives with deletions from the right end (series R) decreased gradually as the deletions extended (Figure 1C). Thus, no distinct region responsible for efficient replication of pW1–1 exists in the right half of the fragment.

A Short Segment Required for Efficient Replication of pW1–1

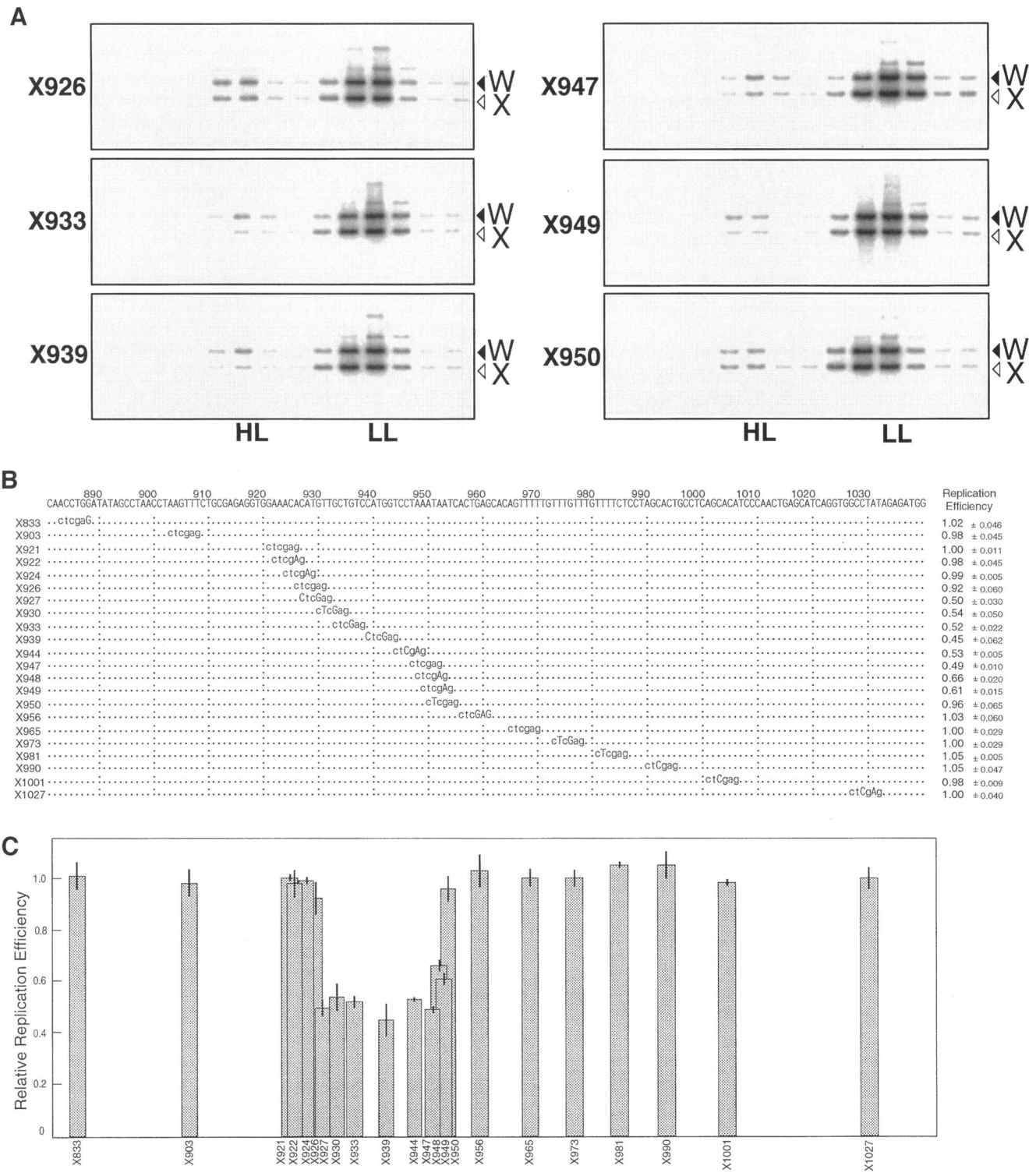
To delimit more precisely the region required for replication of pW1–1, various 6-bp segments in the region between positions 895 and 1025 were replaced by *Xho*I recognition sequence (CTCGAG) (Figure 2B). Replication of several substitution derivatives in 293S cells cotransfected with an internal control pWNS1402 is shown in Figure 2A. Fractions of the substitution derivatives X926 and X950 shifted to the HL density (the lower bands in X926 and X950 in Figure 2A) were almost the same as that of the control plasmid (the upper bands). On the other hand, the fractions of X933, X939, X947, and X949 in the HL density were much smaller than that of the control plasmid. The replication efficiencies of substitution mutants relative to the efficiency of the control plasmid are summarized in Figure 2C. A cluster of substitutions of X929 to X949 resulted in decrease of replication efficiency to about one-half of that of the parental plasmid, but the other substitutions did not affect the efficiency. Base changes from positions 926 to 931 (X926) did not affect the replication efficiency. On the other hand, base changes from positions 928 to 932 (X927) reduced the efficiency to about 50%. Therefore, the left border of the segment required for efficient replication is at position 932 (inclusive). Similarly, from the results for X949 and X950, the right border was deduced to be at position 949. From these results, we conclude that the 18-bp segment from positions 932 to 949 is required for the efficient replication of pW1–1. Reduction in the replication efficiency caused by base changes at positions 948 and 949 (X948 and X949) were smaller than the decrease caused by the other substitutions in the cluster, suggesting that these positions have less importance than the other bases in the cluster for efficient replication of the fragment.

Replication-enhancing Activity of the 18-bp Segment

To examine whether the 18-bp segment has autonomous replication activity in human cells, a pUC119-derivative with 10 tandem copies of synthetic oligonucleotides (RE1 fragment) from positions 921 to 953, pUCRE1$_{10}$, was introduced into 293S cells with an internal control pW1–1. As shown in Figure 3, pUCRE1$_{10}$ DNA recovered in the HL density (the lower band) was much less when compared with the control DNA (the upper band). The replication efficiency of pUCRE1$_{10}$ was less than 2% of pW1–1, which was as low as the level of the vector without insertion. These results showed that the segment alone does not promote autonomous replication. On the other hand, when a single copy of RE1 fragment was inserted into WΔ1402 with a deletion from positions 1 to 1402, which replicated at 50% efficiency of pW1–1, the insertion increased the replication efficiency to the level of pW1–1 (Figure 4). This result suggested that the activity of the 18-bp segment in replication is dependent on the presence of other regions of pW1–1.

To examine the effect of additional copies of the RE1 fragment on replication of pW1–1, 10 copies of the

Figure 2 (facing page). Effect of *Xho*I-linker substitution on replication. Various 6-bp natural sequences from positions 833 to 1032 in pWN706S1075 were replaced with an *Xho*I linker (CTCGAG). Replication of plasmids in 293S cells was examined as described in Figure 1 except that the extrachromosomal DNA was digested with *Dra*I and *Not*I. Representative results of hybridization with a ^{32}P-labeled *Dra*I–*Hin*dIII fragment (1.3 kb) of pUC119 as the probe are shown (A). The names of substitution derivatives are shown on the left of the panels. Positions of the 2.7-kb *Dra*I–*Not*I fragment derived from pWNS1402 (W) and the 2.0-kb *Dra*I–*Not*I fragments from substitution derivatives (X) are shown on the right of the panels.



(Figure 2 cont.) Positions of the HL and LL densities are shown below the panels. (B) The nucleotide sequences from position 881 to 1040 of pW1–1 are shown on the top and the positions of *Xho*I substitutions are shown below the natural sequences. Bases substituted for the natural sequences are indicated by the lower case letters. The names of the substitution derivative designate the position of the first nucleotide of the created *Xho*I recognition sites. (C) Vertical bars indicate replication efficiencies of *Xho*I-linker substitution derivatives relative to the parental plasmid calculated as described in Figure 1. Thin bars show standard deviations obtained from more than three independent experiments.

fragment were inserted into pWX933 at the *Xho*I site. Replication efficiency of the plasmid with the insertion in either direction was more than threefold higher than that of pWX933 (Figure 4). In contrast, insertion of 10 copies of the corresponding fragment with the *Xho*I substitution (RX1 fragment) had no effect on the plasmid replication (Figure 4). These results showed that the 18-bp segment has an enhancing activity in replication. When 10 copies of the RE1 fragment were inserted at the multicloning site or the position 9999 in pWX933, the insertion resulted in an increase in the replication efficiency to the same extent as did the insertion at the position 933 (Figure 4), showing that the enhancement is independent of the relative location of the 18-bp segment.

To test whether the RE1 fragment could stimulate replication of human chromosomal fragments other than W1–1, 10 copies of the fragment were inserted into plasmid p4A5 that had been selected as an HER (high efficiency replicating) segment and into p1–205, p1–213, and p5–205, which were not selected as HER (Masukata *et al.*, 1993). As presented in Figure 5, the plasmids with the insertion displayed two- to fourfold higher replication efficiencies than those without insertion. Therefore, we concluded that the 18-bp segment has a replication-enhancing activity in human cells. The 18-bp segment is designated as replication enhancing element: REE1.

Silencing of Transcription by REE1

It has been known that specific nucleotide sequences enhancing replication of a subset of *S. cerevisiae* ARSs

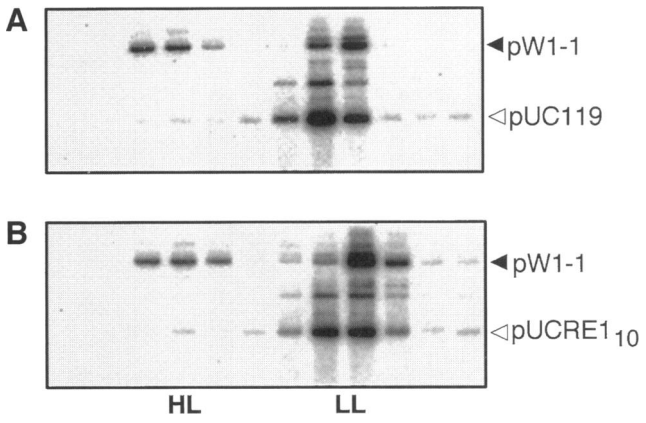


Figure 3. Replication of a pUC119 derivative carrying tandem copies of RE1 fragment. 293S cells were transfected with either a pair of pUC119 and pW1–1 (A) or pUCRE1$_{10}$ and pW1–1 (B). Autonomous replication of plasmids was examined as described in Figure 1 except that the extrachromosomal DNA without linearization by restriction enzymes was examined. The positions of closed-circular molecules are shown on the right of the panel, and the positions of HL and LL densities are shown below the panel.

are the binding sites for transcriptional regulatory factors, such as ABF1 (Shore *et al.*, 1987; Buchman *et al.*, 1988; Marahrens and Stillman, 1992). We examined the effect of REE1 on transcription in human cells, by use of a plasmid pGLPE, in which the SV40 early promoter segment with the transcriptional enhancer was placed upstream of the luciferase reporter gene. The plasmid carrying single or 10 copies of the RE1 fragment at the site 2.7 kb upstream from the promoter was examined for expression of the reporter gene in human 293S cells. The results presented in Figure 6A showed that insertion of a single copy of the RE1 fragment into the plasmid resulted in reduction of the luciferase activity to about 50% (pGLPE-RE1). By insertion of 10 tandem copies of the fragment, in either direction, the luciferase activity was further reduced to about 20% (pGLPE-RE1$_{10A}$, pGLPE-RE1$_{10B}$ in Figure 6A). On the contrary, insertion of the *Xho*I substitution fragment (RX1) had no effect on the luciferase activity (pGLPE-RX1, pGLPE-RX1$_{10A}$, and pGLPE-RX1$_{10B}$ in Figure 6A). Because the copy number of the plasmid in the transfected cells was not significantly changed by the insertion (our unpublished results), the reduction of the luciferase activity was due to repression of transcription from the promoter. These results suggest that REE1 has an activity to repress transcription from the SV40 early promoter.

Because pGLPE contained both the SV40 promoter and the transcriptional enhancer, the reduction of the efficiency of transcription by the presence of the RE1 fragment could result from repression of the promoter activity or from interference with the function of the enhancer. To distinguish between these possibilities, we used pGLP, which deleted the transcriptional enhancer segment of pGLPE. The results in Figure 6B showed that the insertions of single and 10 copies of the RE1 fragment resulted in reduction of the luciferase activity to 40% and 5% of that of pGLP, respectively. The repression was not observed for pGLP derivatives with the RX1 segment at the same site. These results suggest that REE1 has a transcriptional silencer activity. It was observed that transcription from a promoter present near the REE1 in W1–1 fragment was also repressed by the presence of the REE1 sequence (our unpublished results).

To test whether the transcriptional repression by REE1 needs the same nucleotide sequence as that for replication enhancement, effects of various *Xho*I-linker substitutions on transcriptional repression were examined. As shown in Figure 7, the segment carrying the X926 or X950 substitution caused reduction of the luciferase activity to about 50%, which was the same level caused by the segment without substitution (pGLPE-W). In contrast, the segments with X927, X930, X933, X939, X944, and X947 substitutions caused only a slight decrease. For substitutions X948 and X949, the luciferase activity was at an intermediate

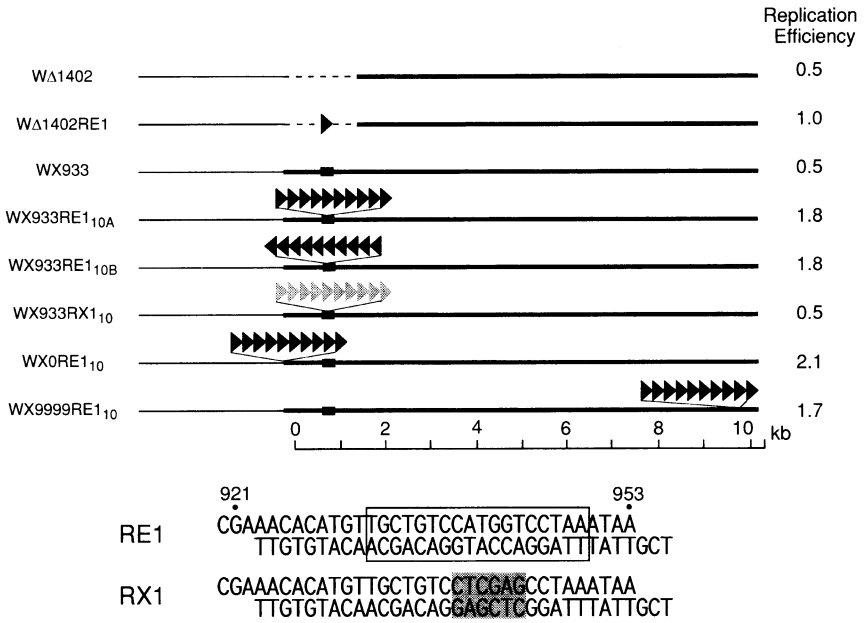


Figure 4. Stimulation of replication of pW1–1 by insertion of tandem copies of RE1 fragment. Replication efficiencies of derivatives of pW1–1 carrying a single or 10 tandem copies of the RE1 or RX1 fragment were determined as described in Figure 1 and the relative values compared with pW1–1 are given on the right. Schematic structures of pW1–1 derivatives are shown with thick and thin lines representing the human fragment and the pUC119 vector, respectively. A broken line shows the region deleted. Solid or shaded triangles respectively represent copies of RE1 or RX1 fragment, the nucleotide sequences of which are shown at the bottom of the figure. The 18-bp segment in the RE1 sequence is indicated by the thin line box and the *Xho*I substitution in RX1 is shown by the shaded square. The fragment inserted in pWΔ1402RE1 contains the sequence from positions 927 to 949 as described in MATERIALS AND METHODS. X933RE1$_{10A}$ and X933RE1$_{10B}$ contain 10 copies of RE1 fragment in the natural and opposite orientations, respectively.

level between those for pGLPE-W and pGLPE-X947 (Figure 7). Together with the results presented in Figure 2C, we conclude that the nucleotide sequences required for transcriptional repression are the same as those for enhancement of replication of chromosomal fragments.

DISCUSSION

We have previously identified human chromosomal fragments that replicate autonomously in human cells at higher efficiencies than randomly cloned chromosomal fragments (Masukata *et al.*, 1993). Here, we showed that the efficient replication of a chromosomal fragment W1–1 was due to the presence of a specific 18-bp sequence, REE1. This is the first demonstration of the presence of a specific nucleotide sequence involved in autonomous replication of human chromosomal fragments.

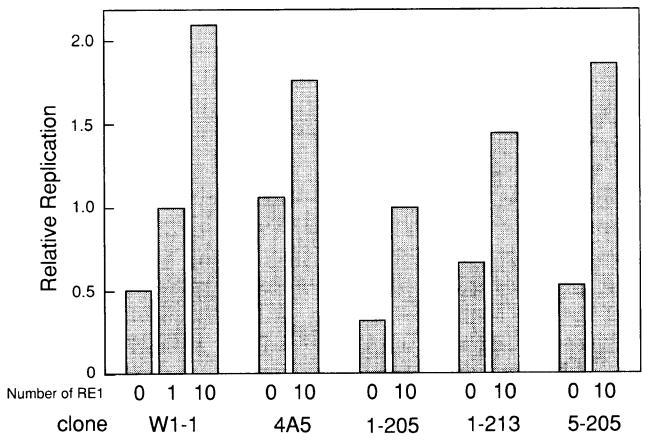


Figure 5. Effect of REE1 on replication of various human chromosomal fragments. Ten copies of RE1 fragment were inserted at the multicloning sites in p4A5, p1–205, p1–213, and p5–205, which carry various human chromosomal fragments (Masukata *et al.*, 1993). Replication efficiencies relative to pW1–1 were determined as described in Figure 1. Plasmids pWX933 and pWX0RE1$_{10}$ were used as the derivatives of pW1–1 without RE1 and with 10 copies of RE1 fragment, respectively.

The elimination of REE1 caused a decrease in the replication efficiency of the W1–1 fragment. Inversely, insertion of extra copies of REE1 into the W1–1 fragment showed stimulation of replication. These results may imply that REE1 would function as a replication origin or a part of an origin. However, because REE1 itself did not display any autonomous replication activity, REE1 alone does not act as an origin of replication. The presence of other regions of W1–1 fragment is necessary for the function of REE1 in replication. The fact that REE1 stimulated replication of randomly cloned chromosomal fragments suggests that the nucleotide sequences of the flanking region are not crucial for the stimulation. The stimulation of replication by REE1 was detectable in the presence of the flanking segment longer than 4 kb (our unpublished results). Because such long DNA segments have certain levels of replication activity, we conclude that REE1 has a replication-enhancing activity.

Because pW1–1 derivatives lacking REE1 still replicate at about 50% of the efficiency of the parental plasmid, regions outside REE1 should be responsible for the remaining replication activity. Deletions outside of REE1 in the W1–1 fragment, particularly in the right half, decreased the replication efficiency in proportion to the length of deletions. Because randomly

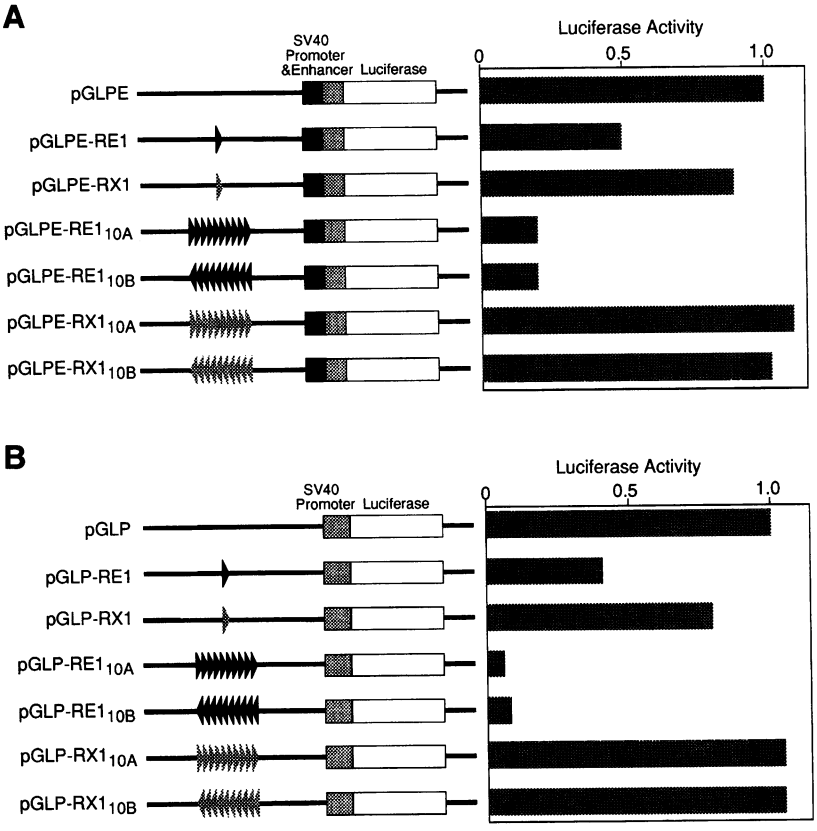


Figure 6. Effect of REE1 on SV40 promoter activity. 293S cells were transfected with a derivative of pGLPE (A) or pGLP (B) together with pMiwZ containing the β-galactosidase gene for normalization of transfection efficiency and with pUC119 as a carrier. At 36 h after transfection, the luciferase activity of cell extracts was measured as described in MATERIALS AND METHODS. Schematic structures of the upstream region of the luciferase reporter gene are shown on the left. A solid or shaded triangle shows an RE1 or RX1 segment, respectively, inserted at the site 2.7 kb upstream of the SV40 early promoter. The relative enzyme activities compared with those of pGLPE or pGLP were obtained and the averages from three independent experiments are presented on the right. The luciferase activity of pGLPE-transfected cells was approximately 10 times higher than that of pGLP-transfected cells.

cloned human chromosomal fragments have been shown to replicate autonomously in human cells at higher efficiencies than bacterial DNA, certain sequences abundant in human chromosome might be involved in the replication (Heinzel *et al.*, 1991; Masukata *et al.*, 1993). Loss of such sequences may cause the decrease in the replication efficiency of the W1–1 fragment by deletions outside of REE1. The 3-kb region from position 7300 to the right end appears to contain abundant nucleotide sequences in the human chromosome, because hybridization of the *Hin*dIII-digested human genomic DNA with a probe for the region gave strong smearing signals ranging from about 1 to 10 kb (our unpublished results). This region is very rich in AT content (more than 75% in average) and has more than 50% homology with the long terminal repeat of human endogenous retrovirus (Ono *et al.*, 1986) and the region downstream of the human β-globin gene containing a family of long interspersed segments, LINE-1 (Hattori *et al.*, 1985). From calculation of the free energy (ΔG) required for unwinding of duplex DNA, the right half of the W1–1 fragment was expected to contain several regions that could be unwound easily. Although such an easily unwound region is suggested to be required for the function of certain yeast ARSs (Natale *et al.*, 1993), elimination of the low ΔG regions from the W1–1 fragment did not cause any greater decrease in the replication efficiency than deletions of the other regions (Figure 1). Thus, the easily unwinding regions in the W1–1 fragment do not appear to contribute largely in the autonomous replication.

It has not been shown whether autonomous replication activity is relevant to the initiation of chromosomal replication in mammalian cells. When we examined the temporal order of replication within a 320-kb region of human chromosome 10, the earliest replicating segments, which are assumed to contain or to be adjacent to the replication origins, had autonomous replication activity two or threefolds higher than other fragments (Ogawa and Masukata, unpublished observation). These results suggest possible association of chromosomal replication origins with autonomous replication activities in human cells. It might be possible that the initiation of chromosomal replication from preferred loci could be due to the presence of a specific sequence element like REE1, which enhances autonomous replication two or threefold.

We found that REE1 had an activity to repress transcription from the SV40 early promoter. Because the promoter activity was repressed by insertion of REE1, in either orientation, at a distant site 2.7 kb upstream of the promoter, we conclude that REE1 has a tran-

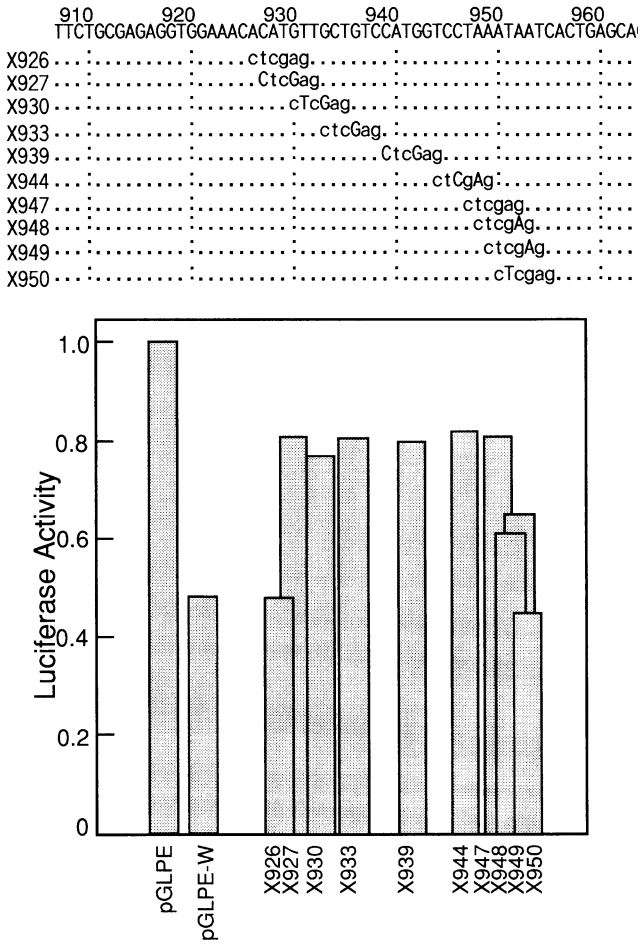


Figure 7. Effect of *Xho*I-linker substitutions on transcriptional silencing. The fragments from positions 706 to 1075 of pW1–1 and of *Xho*I-substitution derivatives were inserted at the site 2.7 kb upstream of the SV40 promoter in pGLPE. The luciferase activity of the transformed cells was measured as described in Figure 6.

scriptional silencer activity. The nucleotide sequences required for repression of transcription, including the two right most bases that are less important, are exactly the same as those for enhancement of replication (Figures 2 and 7), suggesting that the same property of REE1 is responsible for these two different activities. The activity of REE1 in DNA replication and regulation of transcription may be mediated by either formation of an unusual secondary structure or interaction with a specific protein (or proteins). Because the nucleotide sequence of REE1 (Figure 4) does not appear to form any unique DNA structure, it seems likely that a certain protein(s) that binds to REE1 participates in the function of the element. We have found some nuclear proteins interacting specifically with REE1 by a gel mobility shift assay (our unpublished results). Protein factors that bind to REE1 could participate in enhancement of replication and regulation of transcription through interactions with proteins involved in replication and transcription or through alterations in the chromatin structures.

Our finding that REE1 responsible for enhancement of replication of human chromosomal fragments is also involved in repression of transcription may suggest that REE1 serves as a link between the initiation of replication and transcriptional regulation. Involvement of transcription factors in replication of various animal viruses has been described (reviewed in DePamphilis, 1993). In *S. cerevisiae*, some components of the origin recognition complex recognizing the ARS consensus sequence are involved in silencing of transcription in the mating type controlling locus (Bell *et al.*, 1993; Foss *et al.*, 1993; Li and Herskowitz, 1993; Micklem *et al.*, 1993). In addition, the ABF1 protein that binds to replication enhancer elements in a subset of yeast ARSs is also involved in transcriptional regulation of various genes (Shore *et al.*, 1987; Buchman *et al.*, 1988; Marahrens and Stillman, 1992). Thus, it seems likely that DNA replication and regulation of transcription share some common reactions mediated by protein factors that recognize the specific sequences in the replication origins. The presence of REE1 in human chromosomal fragments may suggest that recognition of a specific sequence by a transcription factor might be involved in replication of human chromosome. The role of REE1 in replication in human cells seems to resemble to that of the yeast ABF1 binding site that enhances replication, although the overall structure of human replication origins remains to be identified.

Data base analysis revealed that REE1 has a homology in nucleotide sequence with SRE (serum response element) (Figure 8), which is involved in induction of the human and mouse c-*fos* genes in response to serum factors (Treisman, 1986). SRE has been shown to interact with at least two cellular factors, a serum response factor SRF (Treisman, 1986) and a transcription factor YY1 (Gualberto *et al.*, 1992). REE1 contains a 9/10 match to the consensus sequence, including the CCAT core sequence, for YY1 binding (Figure 8) (Lu *et al.*, 1994; Shrivastava and Calame, 1994). It is therefore possible that YY1 recognizes REE1 that has a transcriptional silencer activity. YY1 is shown to be involved in transcriptional repression of certain genes and activation of some other genes (Shrivastava and Calame, 1994). YY1 that could interact with REE1 may function in both silencing of transcription and enhancement of replication. It has been shown that the properties of YY1 in transcriptional repression or activation and DNA bending resemble those of the yeast ABF1 and RAP1 proteins, which are involved in initiation of DNA replication as well as in regulation of transcription (Natesan and Gilman, 1993). Since a possible YY1 binding site appears to be only a part of the REE1, binding of other factors to REE1 might be also

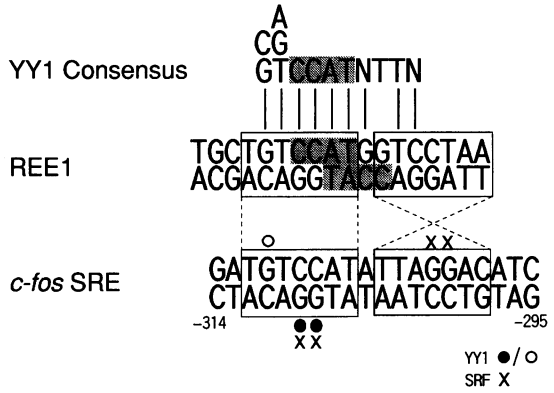


Figure 8. Comparison of nucleotide sequences of REE1 with SRE and the consensus sequence for YY1 binding. The nucleotide sequences of REE1 and the SRE upstream of the human c-*fos* (from positions −314 to −295) (Treisman, 1986) and the consensus sequence for YY1 binding (Lu *et al.*, 1994) are shown. The bases identical between the YY1 consensus sequence and REE1 are indicated by vertical lines and the shaded regions show the core sequence for YY1 binding. The segments identical between REE1 and SRE are indicated by thin line squares. Positions of the methylation interference with the binding of YY1 (●) and SRF (×) are also indicated (Gualberto *et al.*, 1992; Treisman, 1986). An open circle indicates partial interference with binding of YY1.

required for enhancement of replication and repression of transcription. Characterization of REE1 binding factors should be helpful to understand the functions of REE1 in stimulation of replication and repression of transcription in human cells.

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