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Deletion Analysis of Minimal Sequence Requirements for Autonomous Replication of *ors8*, a Monkey Early-Replicating DNA Sequence

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Abstract We have generated a panel of deletion mutants of *ors8* (483 bp), a mammalian autonomously replicating DNA sequence, previously isolated by extrusion of nascent monkey (CV-1) DNA from replication bubbles active at the onset of S phase. The deletion mutants were tested for replication function by the *Dpn*I resistance assay, in vivo, after transfection into HeLa cells, and in vitro. An internal fragment of 186-bp that is required for autonomous replication function of *ors8* was identified. This fragment, when subcloned into pBR322 and similarly tested, was capable of autonomous replication in vivo and in vitro. The 186-bp fragment contains several repeated sequence motifs, such as the ATTA and ATTTAT motifs, occurring three and five times, respectively, the sequences TAGG and TAGA, occurring three and seven times, respectively, two 5′-ATT-3′ repeats, a 44-bp imperfect inverted repeat (IR) sequence, and an imperfect consensus binding element for the transcription factor Oct-1. A measurable sequence-directed DNA curvature was also detected, coinciding with the AT-rich regions of the 186-bp fragment.

Key words: *ors*, replication origin, minimal origin, deletion analysis, episomal replication, in vitro replication

We have previously isolated and cloned, in pBR322, monkey (CV-1) DNA-enriched 10^3- to 10^4-fold for nascent sequences that are replicated at the onset of S phase [Kaufmann et al., 1985]. Approximately 50% (17 of 30) of the origin-enriched sequence (*ors*) clones that have been examined, act as episomal origins of replication, when transfected into HeLa cells, and tested by *Dpn*I resistance, bromodeoxyuridine (BrdUrd) substitution, and electron microscopic examination of replication bubbles [Frappier and Zannis Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]. Seven of the *ors*, *ors1, 3, 8, 9, 10, 11,* and *12* have also been shown to function as plasmid origins of DNA replication in an in vitro replication system that uses HeLa cell extracts [Pearson et al., 1991; Price et al., 1992; Zannis-Hadjopoulos et al., 1992]. Sequence analysis has revealed that the *ors* contain sequence and structural characteristics often associated with replication origins, such as AT-rich regions, inverted repeat (IR) sequences, bent DNA, ARS consensus sequences of yeast, the consensus for scaffold attachment regions (SAR) of *Drosophila*, and various eukaryotic transcriptional regulatory elements [Rao et al., 1990].

ors8 (483 nucleotides in length; ≤ 5 copies per haploid CV-1 genome) [Zannis-Hadjopoulos et al., 1985] is replicated in the early part of S phase [Zannis-Hadjopoulos et al., 1988]. When present on plasmids, it has been shown to act as an episomal DNA replication origin when transfected into mammalian cells [Frappier and Zannis Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991] and in vitro [Pearson et al., 1991]; in both systems, it has been mapped by EM as the initiation site of replication [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1994]. In this study, we have identified by deletion mutagenesis the minimal sequence in *ors8* that is necessary for its function as an episomal origin of replication.

METHODS

Cells and Plasmids

HeLa cells (monolayers) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). All plasmids were propagated in *Escherichia coli* HB101, as previously described [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zan-

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nis-Hadjopoulos, 1991]. Plasmid DNAs were isolated by the alkaline lysis method, as described by Pearson et al. [1991] and by Maniatis et al. [1982].

ors8 Deletion Mutants by Subcloning and Bal31 Exonuclease Digestion

The subfragments of *ors8* plasmid DNA: *Hinf*I–*Hae*III (96–bp), *Hae*III–*Hinf*I (438-bp), *Rsa*I–*Hae*III (192–bp), *Hae*III–*Fok*I (287–bp), *Hae*III–*Nde*I (156–bp), and *Nde*I–*Hae*III (378–bp) were excised and subcloned into the *Nru*I site of pML-2 (a gift from Dr. J. Hassell), as described by Kaufmann et al. [1985]. To generate the internal deletion mutants, *ors8*–pML2 plasmid DNA was linearized by digesting with *Nde*I and then subjected to timed digestion by *Bal31* (BRL; 44 U/ml) for 0.5, 1, 1.5, and 2 min. Aliquots withdrawn at the various time points were diluted 1:1 with an equal volume of stop solution (15 mM EDTA, 0.2% SDS). When all the time points had been collected, the samples were diluted with an equal volume of water, extracted with an equal volume of equilibrated phenol, then with ether, and precipitated by the addition of 2 vol of absolute ethanol. The pellets of the digestion products were then resuspended in a ligation mixture [Maniatis et al., 1982] containing 10 units of ligase (BRL), incubated for 16 hr at room temperature, and then used to transform *E. coli* HB101 cells, as previously described [Kaufmann et al., 1985]. Colonies of isolated transformants were picked, grown as minipreps [Maniatis et al., 1982], and screened by digestion with selected restriction enzymes for determination of the extent of the internal deletion. The size of the deletion mutant plasmids was determined by electrophoresis on polyacrylamide gels, as compared with appropriate size marker standards, and verified by sequencing [Landry and Zannis-Hadjopoulos, 1991]. Finally, the *Nde*I–*Rsa*I fragment (186-bp) of *ors8* was subcloned in the *Nru*I site of pBR322 [Kaufmann et al., 1985].

*Dpn*I Resistance Assay

Exponentially growing HeLa cells were transfected with 3–5 μg of each plasmid DNA (intact *ors8* plasmid or the various deletion mutant plasmids) by the calcium phosphate coprecipitation method, as described previously [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]; 48 h later, low-molecular-weight DNA was isolated by the method of Hirt [1967]; the lysates were extracted, concentrated, digested with 2–3 units of *Dpn*I for 1 h at 37°C, and analyzed by Southern blot hybridization [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]. As an internal control for full digestion by *Dpn*I, 400 ng of methylated λ DNA (Pharmacia) was included in all reactions, and the digestion products were verified by ethidium bromide staining (data not shown). The *Dpn*I-digested DNAs were also used to transform *E. coli* for detecting *Dpn*I-resistant plasmid in Hirt supernatant DNA, as previously described [Landry and Zannis-Hadjopoulos, 1991; Vassilev and Johnson, 1988].

In Vitro Replication Assays

In vitro replication was carried out as described by Pearson et al. [1991], with the following modifications: 100 ng of *ors8* plasmid DNA was used as the template in the control reaction, while all the *ors8*-deletion plasmids and the vector (pML-2) plasmid were used on an equimolar basis relative to *ors8*. For quantitative analysis, each reaction was treated as published in Guo et al. [1989] with some modifications. Briefly, the products of the in vitro reactions were first linearized by digestion with *Sal*I, then digested with *Dpn*I, and separated electrophoretically on 1% agarose gels; 200–300 ng of each respective plasmid in linear form (III) was included as marker. Full-length linear forms were visualized by ethidium bromide staining and excised. The gel slices were dissolved in 1 ml of 4 M urea for 15 min at 95°C before the addition of 15 ml of Universol (ICN) and measurement of radioactivity in a scintillation counter [Guo et al., 1989].

Sequence-Directed Curvature (Bent DNA) Assays

The *ors8* DNA used in these assays was generated by polymerase chain reaction (PCR) amplification of *ors8* plasmid, as described previously [Mah et al., 1993]. The *ors8* portion of the plasmid was amplified using external (pBR-specific) primers (+ and −, sequence of nucleotide positions 954–968 of the top pBR322 strand, and 994–979 of the bottom pBR322 strand, respectively). The PCR-generated *ors8* DNA, which included 21 bp and 20 bp of pBR322 sequence on either side of the cloning site (*Nru* I, pBR322 nucleotide position 972), was digested with the enzymes *Dde*I, *Mbo*II, or *Rsa*I, for the bent DNA assays. The deletion mutants ΔB and ΔC, which were subcloned in the *Nru*I site of the vector

pML-2, were also generated by PCR amplification, using the same primers described above. The presence of anomalously migrating fragments within various subfragments of *ors8* (see Table II) and in deletion mutants ΔB and ΔC was tested by two methods. First, *ors8* was digested with either *Dde*I, *Mbo*II, or *Rsa*I, and the resulting fragments were separated on a 2% agarose gel at room temperature. The lanes were then excised, reoriented at a 90° angle relative to the first dimension, cast in a 7% polyacrylamide gel, and electrophoresed at 9°C [Anderson et al., 1986]; 1 μg of a 123-bp ladder *DNA* marker (Gibco/BRL) was included with each sample. The arc of DNA fragments was visualized by staining with ethidium bromide. In the second method, the digested DNA was loaded onto two parallel 4% polyacrylamide gels and electrophoresed at 9°C and 25°C, respectively; the relative mobility of each fragment was measured with reference to the 123-bp ladder marker.

RESULTS

Description of *ors8* Sequence and the Deletion Mutants

The various landmarks of *ors8* are shown at the top diagram of Figure 1. *ors8* is a 483-bp-long DNA sequence (GenBank Accession No. M26221) [Rao et al., 1990], which contains an AT-rich sequence domain, a 44-bp inverted repeat (IR) sequence (12-bp stem, 20-base loop) [Rao et al., 1990], a GCS consensus sequence characteristic of transcriptional elements upstream of β-globin genes [Rao et al., 1990], a scaffold attachment region (SAR-B) consensus sequence [Gasser and Laemmli, 1986], and a region of perfect homology (11/11-bp match) with the yeast ARS (autonomously replicating sequences) consensus [Palzkill and Newlon, 1988].

Selected subfragments of *ors8* were generated by restriction digestion of the DNA with the appropriate enzymes (see Fig. 1) and subsequently subcloned into the *Nru*I site of the plasmid pML-2, as described in Methods, giving rise to the deletion mutants A–F (Fig. 1). In addition, a panel of internal deletion mutants (Δ1, 6, 10, and 12) (Fig. 1) was generated by timed digestion with *Bal31* of *ors8* that had been previously linearized with *Nde*I (see Methods).

Autonomous Replication Assay of the Deletion Mutants

Supercoiled plasmid DNA from each of these constructs was transfected into HeLa cells and its ability to undergo autonomous DNA replication was assayed by the *Dpn*I resistance assay [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991], in order to distinguish input plasmids from plasmids replicated in HeLa cells. The *Dpn*I-digested DNA samples were divided in two halves, one of which was used to transform *E. coli* (Table I), as described previously [Landry and Zannis-Hadjopoulos, 1991; Vassilev and Johnson, 1988], and the other was subjected to Southern blot analysis (Fig. 2). Both assays showed that deletion mutants B, F, and 12 yielded *Dpn*I-resistant material, while mutants A, C, D, E, 1, 6, and 10 did not (Figs. 1, 2; Table I). These results suggested that an internal region of *ors8,* delimited by the *Nde*I restriction site on one side and *Rsa*I on the other, was necessary for autonomous replicating function.

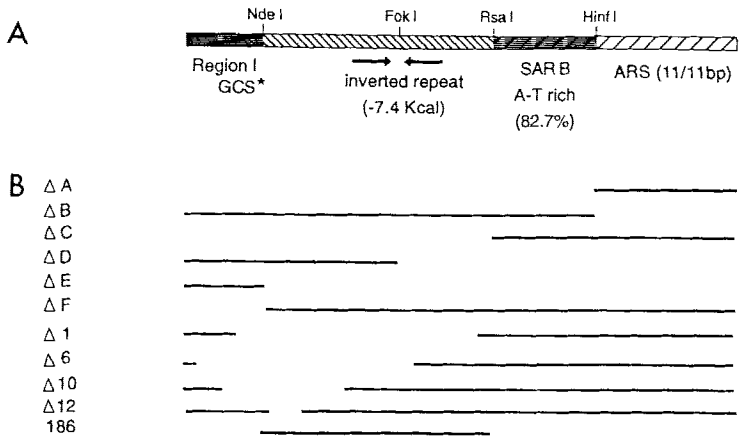
The *Nde*I–*Rsa*I subfragment (186-bp) of *ors8,* heretofore called clone 186, was then subcloned into the *Nru*I site of pBR322 and was subjected to the same analyses as above. The results (Fig. 2; Table 1) showed that clone 186 at 48 h post-transfection yielded *Dpn*I-resistant DNA (Fig. 2A, lane 7), which transformed *E. coli* with an efficiency that was approximately fivefold higher than that of the intact *ors8* plasmid (Table I).

In Vitro Replication of the Deletion Mutants

Analysis of deletion mutant DNA replication in vitro (Fig. 3) yielded results similar to those obtained in vivo, except that in vitro all the deletion mutants were reduced to approximately the same level (20–40%) of replication relative to the intact *ors8* plasmid, while clone 186 replicated with approximately twofold higher efficiency than the intact *ors8* plasmid. The results were the same, regardless of whether in vitro reactions were carried out using equal mass (Fig. 3), or equimolar, amounts of each template DNA (data not shown).

Nucleotide Sequence Features of Deletion Mutant 186

The nucleotide sequence of the 186-bp fragment was re-examined (Fig. 4) in light of its apparent content of sequences essential for the replication origin function. The presence of sev-



C

PLASMID	REGION I	LR.	A/T RICH	ARS/SAR	REPLICATION
ORS 8	+	+	+	+	+
Δ A	-	-	-	+	--
Δ B	+	+	+	-	+
Δ C	-	-	+	+	-
Δ D	+	1/2	-	-	-
Δ E	+	-	-	-	-
Δ F	-	+	+	+	+
Δ 1	+/-	-	+	+	-
Δ 6	-	1/2	+	+	-
Δ 10	-	+	+	+	-
Δ 12	(-30BP)	+	+	+	+
186	–	+	-	-	+
pML2	-	-	-	-	-

Fig. 1. **A:** Schematic diagram of *ors8* (483 bp). The sequence is divided into regions containing the GCS consensus, the inverted repeat, with estimated energy of formation (-kcal), the AT-rich region, and the yeast ARS consensus (see text for details). **B:** Deletion mutants of *ors8*. **C:** Autonomous replication of the deletion mutants, shown in relation to the regions of *ors8* they contain. Replication was assayed by *Dpn*I resistance of transfected plasmids (see Methods).

eral repeated sequence motifs was noted, such as the ATTA and ATTTAT motifs [Boulikas, 1992; Boulikas, 1993a], which occurred three and five times, respectively, the 5′-ATT-3′ sequence [Mastrangelo et al., 1993] repeated twice, and the sequences TAGG and TAGA, which occurred three and seven times, respectively. The inverted repeat (IR) sequence present in *ors8* [Rao et al., 1990], which may assume a cruciform configuration in vivo [Bell et al., 1991], is included in the 186-bp fragment, as is an imperfect consensus element for Oct-1 (ATTTATGCAT) [Iguchi-Ariga et al., 1993, and references cited therein], in which the bases *AT* are inserted in the middle of the consensus.

Bent DNA

ors8 DNA was digested with the restriction enzymes *Dde*I, *Mbo*II and *Rsa*I, and the resulting fragments (Fig. 5) were analyzed for anomalously migrating fragments either by a two-dimensional (2-D) gel assay [Anderson, 1986] or by electrophoresis on parallel polyacrylamide gels at 9°C and 25°C. The relative mobilities of each fragment (Table II) were calculated in relation to the 123-bp ladder marker at 9°C and 25°C. In each digest, bands with a different migration rate at 9°C, by comparison to that at 25°C, were detected and the average percent difference in migration between the two tempera-

TABLE I. Amp-Resistant Colonies Arising From Transformation of *E. coli* HB101 Bacteria With *Dpn*I-Resistant (*Dpn*I-Resistant) Plasmids

	Total no. of *Dpn*I colonies per 3 μg of transfected plasmid DNA	% *Dpn*I-resistant colonies relative to *ors8* plasmid
ors8	16[a] (17,14)[b]	100
ΔA	0	0
ΔB	11 (9,12)	69
ΔC	0	0
ΔD	0	0
ΔE	0	0
ΔF	25 (17,32)	156
Δ1	0	0
Δ6	0	0
Δ10	0	0
Δ12	11 (9,12)	69
186	91 (107,74)	568
pML-2	0	0

[a]Average number of colonies from two experiments.
[b]Each number in the brackets represents the total number of colonies obtained per experiment.

tures was calculated (Table II, and Fig. 5). The average range of difference in relative fragment migration under cold electrophoresis conditions varies from 1.8% (Fig. 5, ΔC) to 4.6% (Fig. 5, *Dde*I), depending on the restriction fragment tested. Thus, the 409-bp *Dde*I fragment of *ors8* migrates 4.6% slower at 9°C than at 25°C, while the *Rsa*I fragment migrates 3% faster at 9°C than at 25°C. The occurrence of increased mobilities of fragments containing poly(dA) · (dT) tracts has been reported [Anderson, 1986, and references cited therein]. Two of the deletion mutants, ΔB and ΔC, were similarly tested for the presence of bent DNA (Fig. 5). The anomalous migration of several of these fragments (Table II) was verified on 2-D agarose–polyacrylamide gels. In this assay, restriction fragments are separated on the basis of their size in the first dimension (agarose gel). The lanes are then excised from the gel, rotated 90° and run in 7% polyacrylamide gels at 9°C (Fig. 6). DNA fragments of the 123-bp ladder form a smooth arc in the second dimension, whereas fragments with bent DNA migrate anomalously relative to molecular weight and therefore are located off the smooth arc formed by random DNA fragments. The 409-bp *Dde*I fragment of *ors8* exhibits anomalous migration when subjected to the 2-D gel assay (Fig. 6). Comparative analysis of the results obtained with the various fragments and deletion mutants (Table II) indicates that the 235-bp fragment, contained between the *Dde*I and *Rsa*I sites, accounts for most of the apparent curvature (anomalous migration) observed in *ors8*. Macroscopically bent DNA arises from oligo (dA) tracts of 3–6 bp in length, repeated in phase with the helix periodicity of 10–11 bp, known as bend elements [Eckdahl and Anderson, 1990, and references cited therein]. The 409-bp *Dde*I fragment of *ors8* contains five $d(A)_3$ bend elements, separated by 10 or 11 bp (nucleotide position 209–265, Fig. 4). This fragment contains the 186-bp *Nde*I–*Rsa*I fragment.

DISCUSSION

We have previously demonstrated that *ors8* (483 bp), obtained by short nascent strand extrusion of monkey (CV-1) DNA from replication bubbles active at the onset of S phase [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985], can function as an origin of DNA replication in autonomously replicating plasmids [Frappier and Zannis-Hadjopulos, 1987] and in a mammalian cell-free replication system [Pearson et al., 1991]. *ors8* is present in less than 5 copies per haploid genome [Zannis-Hadjopoulos et al., 1985] and is contained within a 1.2-kb *Pst*I fragment of CV-1 genomic DNA [Mah et al., 1993]. Primary sequence analysis [Rao et al., 1990] has shown that it contains extensive AT-rich regions that coincide with areas of detectable anomalous migration of the DNA indicative of altered structure, an IR potentially capable of extruding into a cruciform [Bell et al., 1991], AP3 and SAR-B consensus, and a perfect (11/11-bp) yeast ARS consensus sequence (ACS) [Palzkill and Newlon, 1988]. Here, we have generated a panel of deletion mutants and tested them for origin function by the *Dpn*I-resistance assay, in vivo (by transfection) and in vitro. Our analyses indicate that an internal region of 186-bp that comprises the 44-bp IR [Rao et al. 1990], several direct repeats, such as the ATTA, ATTTAT, 5′-ATT-3′, TAGG and TAGA sequence motifs, an imperfect Oct-1 consensus (ATTTATGCAT), and areas of apparent DNA curvature, is crucial for in vivo and in vitro replication function of *ors8*. This 186-bp subfragment, when subcloned into pBR322 and similarly tested, was found capable of autonomous replication in vivo as well as in vitro. The results suggest that a minimal *ori* lies within the 186-bp (*Nde*I–*Rsa*I) fragment of *ors8* and that the sequences lying outside this minimal *ori* seemingly contain elements

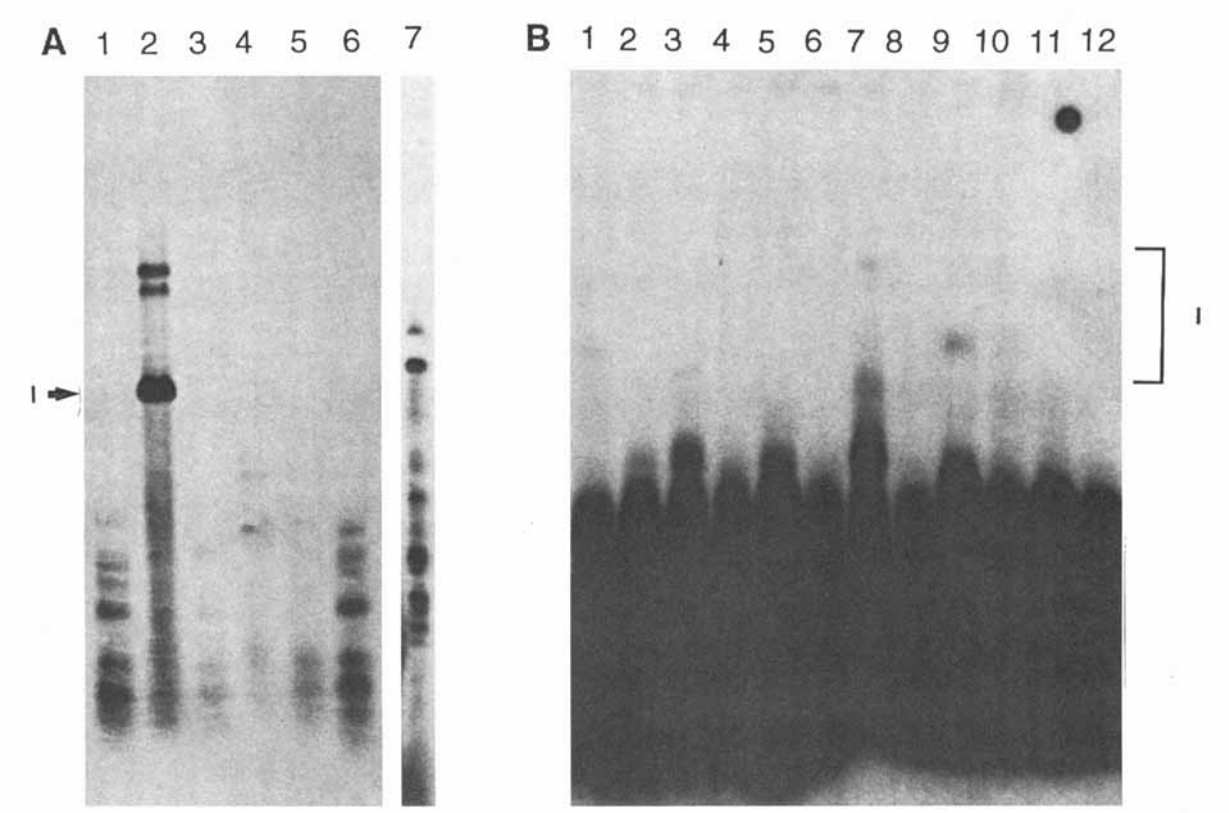


Fig. 2. Autonomous replication assay of the deletion mutants by *Dpn*I resistance. Southern blot analysis (see Methods) **A:** 1 = pML-2; 2 = ors8; 3 = ΔA; 4 = ΔC; 5 = ΔD; 6 = ΔE; 7 = 186. Form I is indicated by the arrows. **B:** 1 = ΔF; 2 = ΔA; 3 = ΔB; 4 = ΔE; 5 = ΔC; 6 = pML-2; 7 = ors8; 8 = ΔD; 9 = Δ12; 10 = Δ10; 11 = Δ6; 12 = Δ1. Form I is indicated by the bracket. The amount of DNA loaded to produce the blot in B is approximately one third of that in A.

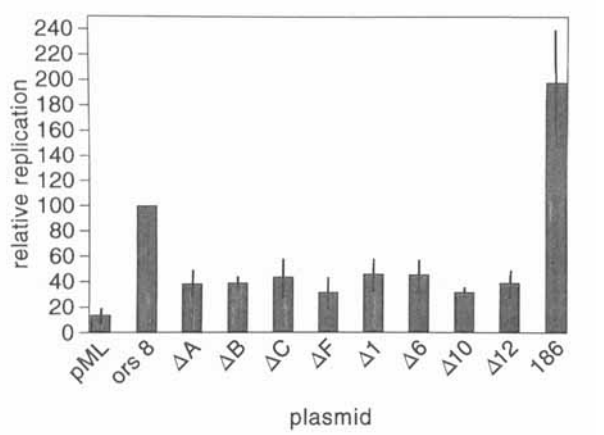


Fig. 3. In vitro replication activity of the *ors8* deletion mutants (see Methods for assay). All data have been plotted relative to the incorporation of the complete *ors8* plasmid. Each bar represents multiple experiments.

that exert a negative effect on the replication of the intact plasmid. Interestingly, deletions B, F, and 12, when tested by the *Dpn*I-resistance assay in vivo, yielded products of equal intensity to the intact *ors8* plasmid. These same deletion mutants, when tested for replication in vitro were reduced to the same extent (20–40%) as the other deletion mutants relative to the intact *ors8* plasmid. This difference most likely reflects the different requirements for replication between the two systems as previously observed [Nielsen et al., 1994; Pearson et al., 1991].

The ATTA and ATTTA motifs, which constitute the core elements recognized by the homeobox domain from species as divergent as flies and humans, frequently occur in the matrix attachment sites of several genes, as well as in several eukaryotic and viral origins of DNA replication [Boulikas, 1992], including the mammalian *ors17, ors24,* and *ors25* [Landry and Zannis-Hadjopoulos, 1991], the replication origin of the human c-*myc* gene [Iguchi-Ariga et al., 1993], and the replication origin of the Chinese hamster *dhfr* gene [Caddle et al., 1990]. Recently, we showed that replicating genomic *ors8* is enriched on the nuclear matrix in early S phase [Mah et al., 1993]. Similar AT-rich repeat motifs have also been found in the minimal replication

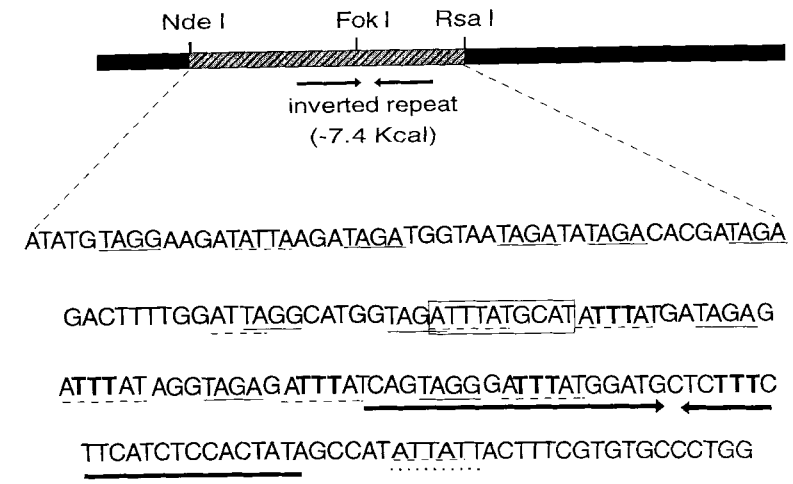


Fig. 4. Sequence detail of the 186-bp deletion mutant of *ors8*. The sequence motifs TAGG, and TAGA (solid underline), ATTA and ATTTAT (dashed underline), the imperfect Oct 1 binding site (boxed), the 5'-ATT-3' repeats (dotted underline) and the five $d(A)_3$ bend elements that are in phase with the helix periodicity (10–11 bp apart, boldface) are indicated.

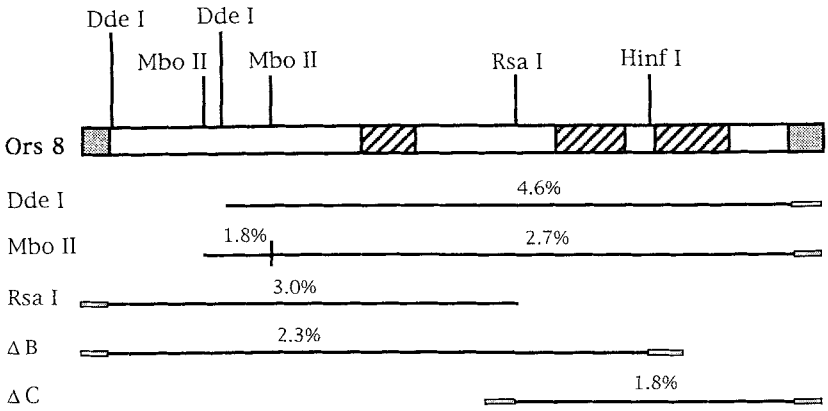


Fig. 5. DNA curvature of *ors8*. The indicated restriction fragments and deletion mutants of *ors8* were run on parallel polyacrylamide gels at 9°C and 25°C (see Methods). Fragments exhibiting anomalous migration are shown along with the percent differences in relative mobility between the two temperatures. Regions of greater than 80% A + T content (diagonal lines) and pBR322 sequences (stippled regions) are indicated.

origin of the 200-kb *Halobacterium* plasmid pNRC100 [Ng and DasSarma, 1993] and other prokaryotes [Eckdahl and Anderson, 1990]. It has been postulated that one possible function of such repeats could be in binding of replication proteins, with the formation of a melted replication complex being facilitated by the AT-rich regions [Eckdahl and Anderson, 1990; Ng and DasSarma, 1993; Kornberg and Baker, 1992].

The presence of transcriptional regulatory elements are a common feature of eukaryotic replication origins and are thought to be implicated in the temporal regulation of replication [reviewed in DePamphilis, 1993]. The 186-bp fragment contains an imperfect consensus binding site for the transcription factor Oct-1; the octamer transcriptional element has been recently suggested as a putative origin for cellular DNA replication [Iguchi-Ariga et al., 1993]. Not included in the 186-bp fragment, but immediately upstream of it, lies the β-globin upstream transcriptional control sequence, CACCC, which is also contained in the portion of the SV40 enhancer that binds AP3 [Rao et al., 1990].

Among the sequence features present in the 186-bp fragment of *ors8* is a 44-bp imperfect IR, whose two branches are 20 bp apart from each other and whose estimated energy of formation is −7.4 kcal [Rao et al., 1990]. IRs, a common feature of prokaryotic and eukaryotic replication origins [Muller and Fitch, 1982; Campbell, 1986; Boulikas, 1993b], have been shown to be

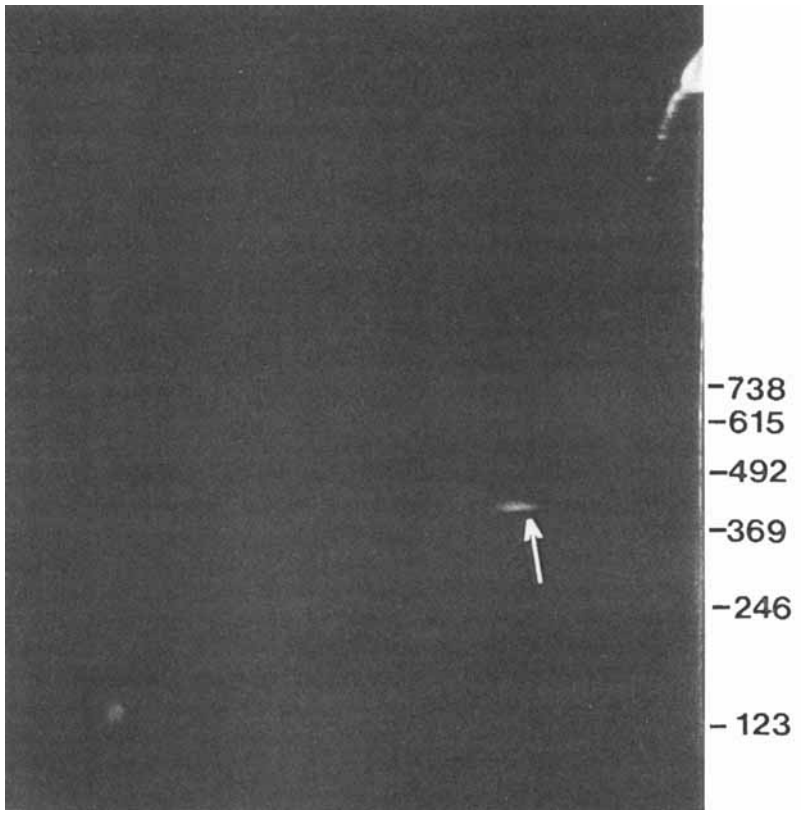


Fig. 6. Anomalous migration of the 409-bp *Dde*I fragment of *ors8* on a 2-D agarose polyacrylamide gel. The *Dde*I digest of *ors8* PCR product (see Methods) was separated together with the 123-bp ladder in the same lane. Electrophoresis in the first dimension was in 2% agarose. The lane was then excised, rotated 90°, and subjected to 7% polyacrylamide electrophoresis at 9°C in the second dimension. The 2-D migration pattern was visualized by staining with ethidium bromide. The 409-bp *Dde*I fragment is indicated (arrow). Marker sizes (in bp) are also indicated.

functionally important for the initiation of DNA replication in plasmids [Masukata and Tomizawa, 1984; Noirot et al., 1990], prokaryotes [Zyskind et al., 1983; Hiasa et al., 1990; Brantl and Behnke, 1992], and eukaryotic viruses [Frisque, 1983; Stow and McMonagle, 1983; Reisman et al., 1985; Weller et al., 1985; Deb et al., 1986; Lockshon and Galloway, 1986; Prives et al., 1987]. We have previously reported that IRs are enriched in monkey [Rao et al., 1990; Zannis-Hadjopoulos et al., 1985; Zannis-Hadjopoulos et al., 1984] and human [Nielson et al., 1994] *ors*. IRs have the potential to form cruciform structures under conditions of torsional strain on the DNA [Panayotatos and Wells, 1981], and the occurrence of cruciforms in vivo has been demonstrated in DNA of prokaryotes [Panayotatos and Fontaine, 1987; Dayn et al., 1992] and the eukaryotic virus SV40, at the viral origin of replication [Hsu, 1985]. We have previously obtained evidence that the IR present in *ors8* is capable of extruding into a cruciform in vivo

TABLE II. Relative Mobility of *ors8* and Its Subfragments on 1-D Gel Electrophoresis at 9°C and at 25°C

Fragment	Fragment size (bp)	Relative mobility (bp) 25°C	Relative mobility (bp) 9°C	Average % difference
ors8	528	530	550	3.7[a]
*Dde*I	42	—	—	—
	77	—	—	—
	409[b]	420	440	4.6[a]
*Mbo*II	106	113	113	0.9[a]
	47	125	123	1.8[a]
	375	265	260	2.7[a]
*Rsa*I	332[b]	335	325	3.0[a]
	196	198	198	0.0[a]
Deletion mutants				
ΔB	449	430	440	2.3
ΔC	218	230	226	1.8

[a]Data from several experiments. [b]Confirmed by two-dimensional gel data.

[Bell et al., 1991] and in vitro [Price et al., 1992; Zannis-Hadjopoulos et al., 1992]. The existence of cruciforms in mammals has been associated with the process of initiation of DNA replication [Zannis-Hadjopoulos et al., 1984; Zannis-Hadjopoulos et al., 1988; Hand, 1978].

Finally, it is interesting to note that the perfect (11/11-bp) yeast ACS [Palzkill and Newlon, 1988] present in *ors8*, which is essential for ARS function in yeast [Van Houten and Newlon, 1990], is not included in the 186-bp fragment of *ors8*. In the plasmid (ΔB) that had the region of *ors8* containing the yeast ACS deleted, the replication efficiency, both in vivo and in vitro, was only diminished by approximately 30%.

To assess the importance of the various sequence elements that have been identified in the 186-bp fragment of *ors8*, we are currently investigating the protein–DNA interactions between this *ors8* subfragment and replication proteins that are purified from HeLa cells.

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