Journal of Cellular Biochemistry 66:87–97 (1997)

Deletion Analysis of *ors12,* a Centromeric, Early Activated, Mammalian Origin of DNA Replication

Richard Pelletier,[1] **David Mah,**[1] **Suzanne Landry,**[1] **Diamanto Matheos,**[1,2] **Gerald B. Price,**[1] **and Maria Zannis-Hadjopoulos**[1,2]*****

[1]McGill Cancer Centre, McGill University, Montréal, Québec, Canada
[2]Department of Biochemistry, McGill University, Montréal, Québec, Canada

Abstract We have generated a panel of deletion mutants of *ors12* (812-bp), a mammalian origin of DNA replication previously isolated by nascent strand extrusion from early replicating African Green monkey (CV-1) DNA. The deletion mutants were tested for their replication activity in vivo by the bromodeoxyuridine substitution assay, after transfection into HeLa cells, and in vitro by the *DpnI* resistance assay, using extracts from HeLa cells. We identified a 215-bp internal fragment as essential for the autonomous replication activity of *ors12.* When subcloned into the vector pML2 and similarly tested, this subfragment was capable of autonomous replication in vivo and in vitro. Several repeated sequence motifs are present in this 215-bp fragment, such as TGGG(A) and G(A)AG (repeated four times each); TTTC, AGG, and CTTA (repeated 3 times each); the motifs CACACA and CTCTCT, and two imperfect inverted repeats, 22 and 16 bp long, respectively. The overall sequence of the 215-bp fragment is G/C-rich (50.2%), by comparison to the 186-bp (33.5% G/C-rich) minimal sequence required for the autonomous replication activity of *ors8,* another functional *ors* that was similarly isolated and characterized. J. Cell. Biochem. 66:87–97, 1997. © 1997 Wiley-Liss, Inc.

Key words: deletion mutants; *ors12;* replication activity; mammalian origin; autonomous replication

We have previously isolated origin-enriched sequences (*ors*) from African green monkey (CV-1) kidney cells by extrusion [Zannis-Hadjopoulos et al., 1981] of nascent DNA in early S-phase from small replication bubbles [Kaufmann et al., 1985]. *Ors* sequences are enriched for inverted repeats (IRs) that have the potential to form cruciform structures in vivo [Zannis-Hadjopoulos et al., 1984, 1985; Pearson et al., 1996]. The *ors*-containing plasmids are capable of transient autonomous replication in vivo, when transfected into monkey (CV-1 and Cos-7) and human (HeLa) cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993] and in an in vitro replication system that uses HeLa cell extracts [Pearson et al., 1991]. Both in vivo and in vitro, replication is semi-conservative, depends on the presence of an *ors*-containing template, and initiates within the *ors* sequence [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991, 1994; Zannis-Hadjopoulos et al., 1992]. One of the functional *ors, ors12* (812-bp), is present in less than 9 copies per haploid genome and has been localized by in situ hybridization to the centromere of six CV-1 chromosomes as well as that of a marker chromosome [Mah et al., 1992]. *ors12* associates with the nuclear matrix in a cell cycle-dependent manner and replicates on the matrix in early- to mid-S phase [Mah et al., 1993]. Restriction mapping analysis of CV-1 genomic DNA has revealed considerable conservation in the arrangement of the different *ors12* copies [Mah et al., 1992]. We have recently shown that *ors12* serves as a chromosomal origin of DNA replication in CV-1 cells (Pelletier et al., manuscript in preparation). Here we demonstrate, by deletion analysis, that an internal fragment of *ors12,* extending from nucleotide 362 to 577, is essential for its autonomous replication activity in vivo and in vitro.

Contract grant sponsor: Medical Research Council of Canada, MT-7965.

*Correspondence to: Dr. Maria Zannis-Hadjopoulos, McGill Cancer Centre, 3655 Drummond Street, Montréal, Québec H3G 1Y6, Canada. E-mail: hadjopoulos@medcor.mcgill.ca

Received 29 January 1997; accepted 11 March 1997

© 1997 Wiley-Liss, Inc.

MATERIALS AND METHODS

Cells and Plasmids

Hela cells (monolayers) were cultured in Dulbecco's minimal essential medium (DMEM)

supplemented with 10% fetal calf serum. *ors12* (812-bp; GenBank Accession no. M26225) plasmid has been previously described [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985; Rao et al., 1990]. pML2-based plasmids were propagated in *Escherichia coli* HB101, while pBluescript-based plasmids were propagated in the DH5αF′ strain, as previously described [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991].

Construction of *ors12* Deletion Mutants

The internal deletion mutants d1(10) and d1(240) were constructed by linearizing the pML-*ors12* plasmid with *BalI*, digesting with *Bal31* exonuclease (BRL; 44U/ml) for 0.5–2 min, filling the ends with the Klenow fragment of DNA polymerase I [Maniatis et al., 1989], and religating them with T4 DNA ligase (New England Biolabs, Beverly, MA), as previously described [Todd et al., 1995]. Deletion mutants dα(238), carrying a 238-bp deletion, and dα(272), carrying a 272-bp deletion, were generated as follows: pML-*ors12* was linearized with *XmaIII* and subjected to digestion by *Bal31* (BRL; 50 U/72 μl) for 10 min; the DNA was then digested with *EcoRI* and *BglII* (New England Biolabs), ends were filled with T4 polymerase, sequenced by the chain termination method [Sanger et al., 1977] to verify the endpoints of each deletion, and the fragments of the desired size (Fig. 1B) were subcloned into the *EcoRV* site of pBluescriptKS(−) vector. Deletion mutant d1 was generated by removing the *BamHI-ScaI* (977-bp) fragment from pML-*ors12*, replacing it with the *BamHI-NruI* (597-bp) fragment of the pML2 vector, and ligating the ends with T4 DNA ligase. Deletion mutant d2 was generated by removing the *ScaI-StyI* (215-bp) fragment from pML-*ors12*, and repairing the ends with the Klenow polymerase. Deletion mutant d3 was constructed by removing the *HindIII-StyI* (1,564-bp) fragment of pML-*ors12*, filling in the end generated by *StyI* with Klenow polymerase and subcloning it into pML2, from which the *HindIII-NruI* (943-bp) fragment had been removed. Similarly, deletion mutant d2/3 was generated by replacing the *HindIII-NruI* (943-bp) fragment in pML2 by the *HindIII-ScaI* (1,349-bp) fragment of pML-*ors12*. For deletion mutant d1/3, the *ScaI-StyI* (215-bp) fragment of *ors12* was excised, the ends were filled with the Klenow polymerase, and it was then subcloned into the *NruI* site of pML2.

Transfections and Autonomous Replication Assays

Each plasmid DNA was prepared by the alkaline lysis method and purified by two successive rounds of CsCl centrifugation [Maniatis et al., 1989]. Supercoiled DNA (5 μg) was transfected into HeLa cells by the calcium phosphate coprecipitation method [Graham and Van der Eb, 1973] and after transfection the cells were grown in media containing bromodeoxyuridine (BrdUrd), as previously described [Landry and Zannis-Hadjopoulos, 1991; Todd et al., 1995). Plasmids were recovered by Hirt's lysis [Hirt, 1967], loaded on CsCl gradients (initial refractive index 1.408) and centrifuged as described previously [Landry and Zannis-Hadjopoulos, 1991]. An aliquot of each fraction was dot-blotted onto a GeneScreen Plus membrane (Dupont, Wilmington, DE), hybridized to ^{32}P-labelled vector sequences (pML2 or pBluescript plasmid DNA), exposed to an imaging plate, and quantitated by densitometry using a Phosphoimager (Fuji BAS 2000, Stamford, CT).

In Vitro Replication

The *ors12* deletion mutants were assayed for replication in vitro as described previously [Pearson et al., 1991; Todd et al., 1995], with the following modifications: (1) pML2 (200 ng) was used as template in the negative control reaction, while all the other plasmids were used in equimolar amounts relative to it; (2) the products of the in vitro reactions were suspended in 16 μl of TE (pH 8.0) total. One fourth of each reaction (4 μl) was kept undigested, and another 4 μl was digested with 0.5 to 2 units of *DpnI* (New England Biolabs) in the buffer provided by the manufacturer, to which NaCl was added to a final concentration of 200 mM. The digestions were carried out for 1 to 2 h at 37°C. Both samples (digested and undigested) were subjected to electrophoresis on 1% agarose gels and the gel was exposed and analyzed on the Phosphoimager (Fuji BAS 2000, Stamford, CT).

RESULTS

Topology of *ors12* and Description of the Deletion Mutants

The salient features of *ors12* (812-bp) are shown in Figure 1A. Briefly, *ors12* contains 168-bp of α-satellite DNA at its 5′-end, three inverted repeats (IRs; free energies of formation, ΔG: −15.6 Kcal, −9 Kcal, and −12.3 Kcal,

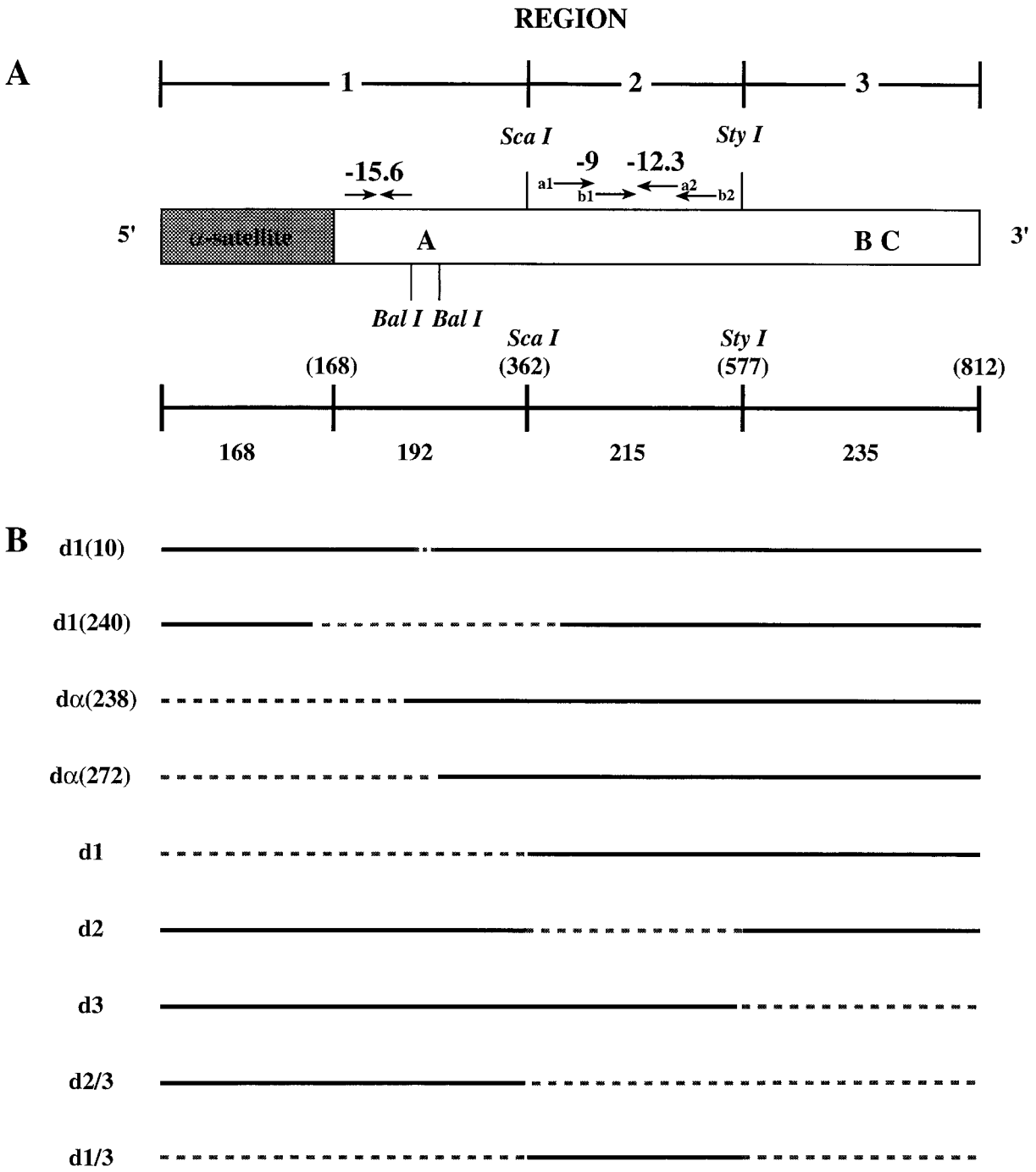

Fig. 1. **A:** Genetic map of *ors12*. The sequence is divided into 3 regions (upper bar) defined by the *ScaI* and *StyI* restriction sites (see text for details). *Ors12* and its most important landmarks are shown (boxed area): the α-satellite repetitive sequence (dark shaded area); the approximate location of the CACCC consensus (A); the yeast (*S. cerevisiae*) ARS consensus (B) and the scaffold attachment region (SAR-T) (*Drosophila*) (C). The position of the three inverted repeats is indicated (inverted arrows), with their estimated free energy of formation (−Kcal). The nucleotide position of the restriction sites used for generating the deletion mutants is also shown (bottom bar). The numbers below the bar indicate the size of the fragments in each region. **B:** Schematic representation of deletion mutants tested in this study. The dashed lines represent the deleted portion in each clone.

respectively), which may form cruciform structures in vivo [Zannis-Hadjopoulos et al., 1984, 1988, 1992], the CACCC consensus sequence (Fig. 1A; element A) of the mouse β-globin enhancer [Dierks et al., 1983], an imperfect match (10/11) to the ARS consensus (Fig. 1A; element B) of yeast (*Saccaharomyces cerevisiae*) [Palzkill and Newlon, 1988], and a scaffold attachment region (SAR-T) consensus of Drosophila (Fig. 1A; element C) [Gasser and Laemmli, 1986]. For generating the deletion mutants, we subdivided *ors12* into three arbitrary regions, defined by the *ScaI* and *StyI* restriction sites (Fig. 1A). Region 1 extends from the 5′-end to nucleotide 362, and comprises the α-satellite repetitive sequence, an IR ($\Delta G = -15.6$ Kcal) and the CACCC consensus sequence; region 2, which is delimited by the *ScaI* (nt 362) and *StyI* sites (nt 577), comprises two IRs ($\Delta G = -9$ Kcal and -12.3 Kcal, respectively); and region 3, which extends from the *StyI* (nt 577) site to the 3′-end (nt 812) of *ors12*, includes an AT-rich region comprising the ARS and the SAR-T consensus.

Autonomous Replication In Vivo

To assess the role played by these various elements in the autonomous replication activity of *ors12* in vivo and in vitro, we constructed a series of mutants in which selected parts of the *ors12* sequence were deleted (Fig. 1B). For each mutant clone, the autonomous replication activity in vivo was evaluated by density shift assays following bromodeoxyuridine (BrdUrd) incorporation [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]. For each set of experiments, a plasmid containing the intact *ors12* (pML-*ors12*, positive control) and a plasmid vector alone (pML2 or pBluescript; negative control) were transfected in separate flasks. The linearity of each gradient was verified by measuring the refractive index of every other fraction (Figs. 2 and 3, ●-●). For all plasmids tested, a peak of unreplicated (LL) DNA was recovered at the top of each gradient (see region including fractions 16–24; Figs. 2 and 3). Additional peaks of replicated, heavy-light (HL) and heavy-heavy (HH) DNA were also obtained for those plasmids that were capable of replicating, indicating one and two or more rounds of replication, respectively. Intact (wild-type) *ors12* (Fig. 2A) was capable of efficient autonomous replication, as newly synthesized plasmid DNA was recovered in the HH part of the gradient (fractions 1–4; Fig. 2A), in agreement with previous results [Frappier and Hadjopoulos, 1987; Landry and Hadjopoulos, 1991; Zannis-Hadjopoulos et al., 1994]. In contrast, also in agreement with previous results [Frappier and Hadjopoulos, 1987; Landry and Hadjopoulos, 1991], neither of the two negative control vectors (pML2, Fig. 2F; pBluescript Fig. 3E) displayed any replication activity, as all plasmid DNA was recovered in the LL form of input DNA.

Mutant d1(10) (Fig. 1B), in which a 10-bp fragment comprising the CACCC consensus sequence [Dierks et al., 1983] was deleted, was recovered as HL and HH DNA (Fig. 2B), demonstrating efficient replication activity. Similarly, mutant d1(240) (Fig. 1B), in which a 240-bp segment comprising the CACCC sequence, the first IR ($\Delta G = -15.6$ Kcal) and part of the α-satellite region [Rao et al., 1990], was deleted, also exhibited episomal replication activity (Fig. 2C), as did deletion mutant d1 (Fig. 2D), in which the entire region 1 was deleted (Fig. 1B). However, when region 2 was deleted from *ors12* (mutant d2, Fig. 1B), the plasmid was unable to support autonomous replication, as no DNA was recovered in either the HL or HH regions of the gradient (Fig. 2E). In contrast, mutant d3, which is missing the entire region 3, spanning the AT-rich region that includes the ARS and SAR-T consensus (Fig. 1A), maintained efficient replication activity, as judged by the substituted DNA (Fig. 3A).

Two double mutants, d2/3 and d1/3, bearing deletions in two out of the three regions of *ors12*, were also similarly assayed. Deletion mutant d2/3 (Fig. 3B), in which regions 2 and 3 were removed, displayed no autonomous replication activity, as no substituted DNA was recovered (Fig. 3B). Occasionally, in assays using this clone, a small amount of DNA was recovered in the HL fractions, suggesting that region 1 alone can support autonomous replication of the plasmid, albeit inefficiently. On the other hand, when region 2 alone was subcloned independently in pML2 (deletion mutant d1/3, Fig. 1B) and similarly assayed, the autonomous replication activity of *ors12* was restored (Fig. 3C), although at a lower efficiency than the full origin (Fig. 2A). Finally, mutant dα(238), carrying a 238-bp deletion that removed the α-satellite portion of *ors12* (Fig. 1B), was not affected in its ability to replicate autonomously, as shown by peaks of HL and HH DNA recovered (Fig.

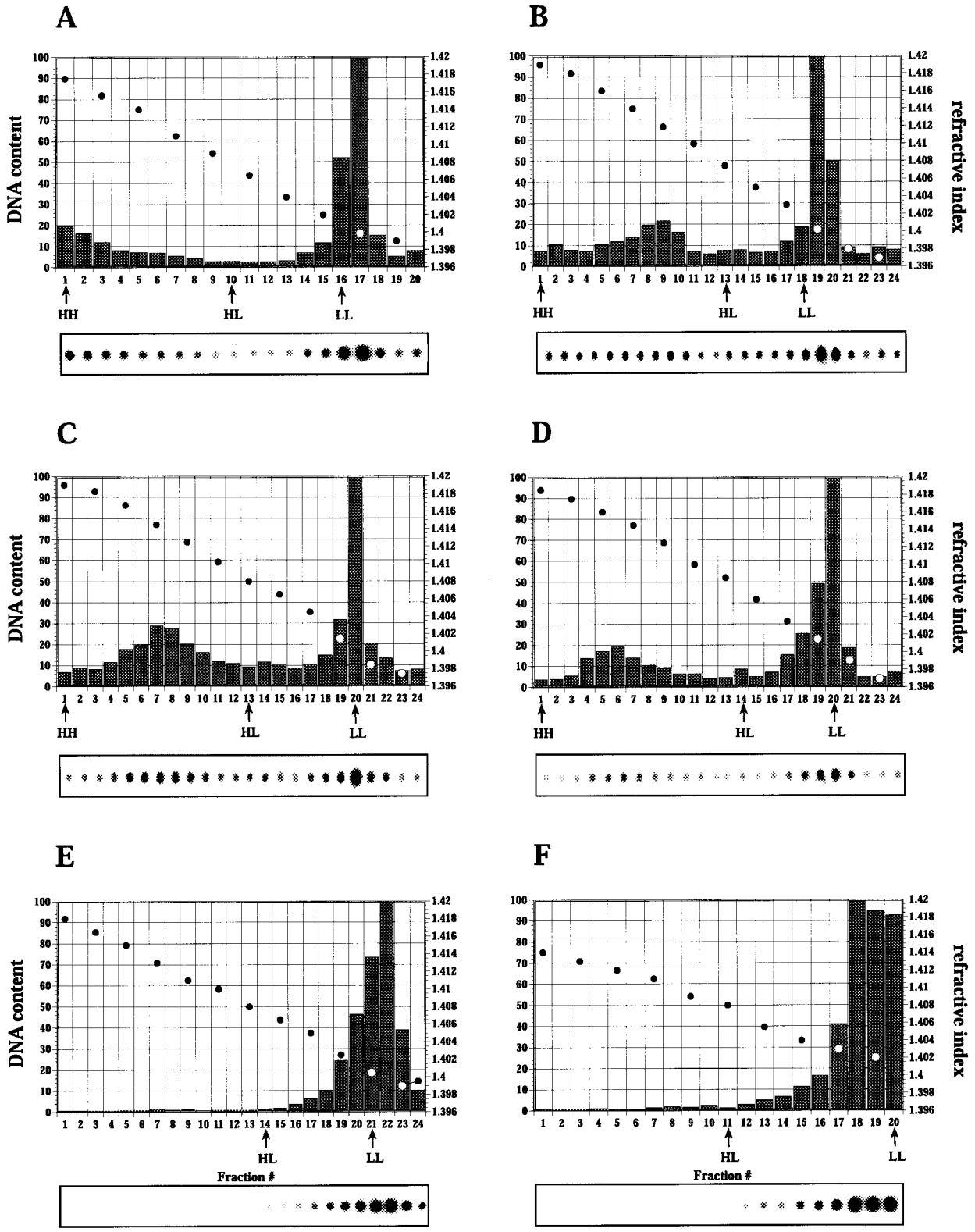

Fig. 2. Autonomous replication assays with pML-*ors12* (**A**), d1(10) (**B**), d1(240) (**C**), d1 (**D**), d2 (**E**), and pML2 (**F**) plasmids. HeLa cells were transfected (see Materials and Methods) with each plasmid DNA and BrdUrd (12.5 µg/ml) was added 24 h after transfection. Hirt's lysates were collected 40 to 48 h later, and banded on native CsCl gradients. Between 20 and 24 fractions were collected from each gradient, dot blotted, and the membrane was hybridized to ^{32}P-labelled pML2 sequences (for pML-*ors12* and pML2 clones), or to ^{32}P-labelled pBluescript sequences (for clone dα(238) and pBluescript). For each gradient, the refractive index of every other fraction was taken (●-●). The histograms generated by densitometry scanning of each dot, representing the DNA content of each fraction, are plotted (arbitrary units) above the corresponding dots. The positions of each density peak (HH, HL, and LL) are indicated.

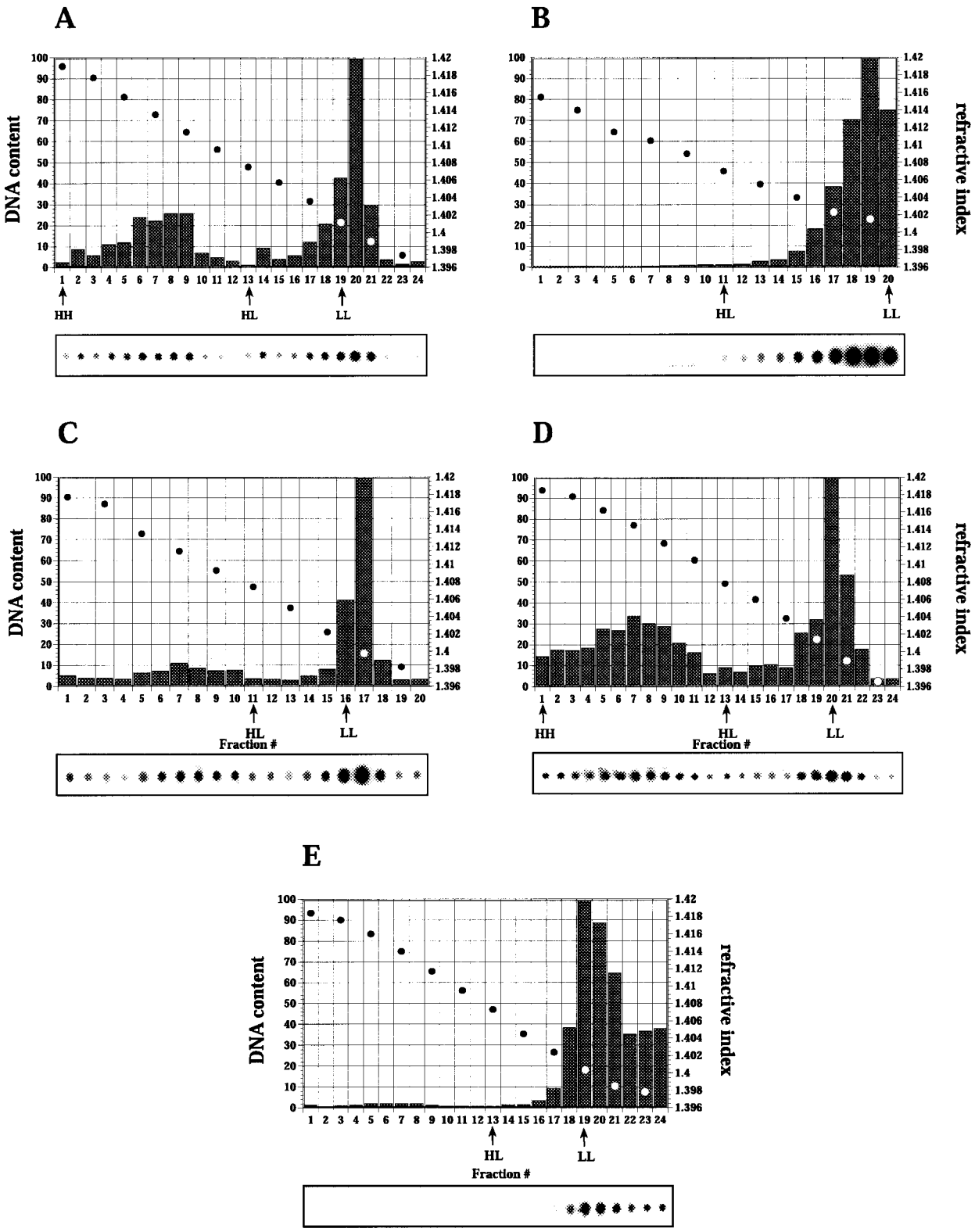

Fig. 3. Autonomous replication assays with d3 (**A**), d2/3 (**B**), d1/3 (**C**), dα(238) (**D**), and pBluescript-KS(+) (**E**) plasmids. The assays were performed as in Figure 2.

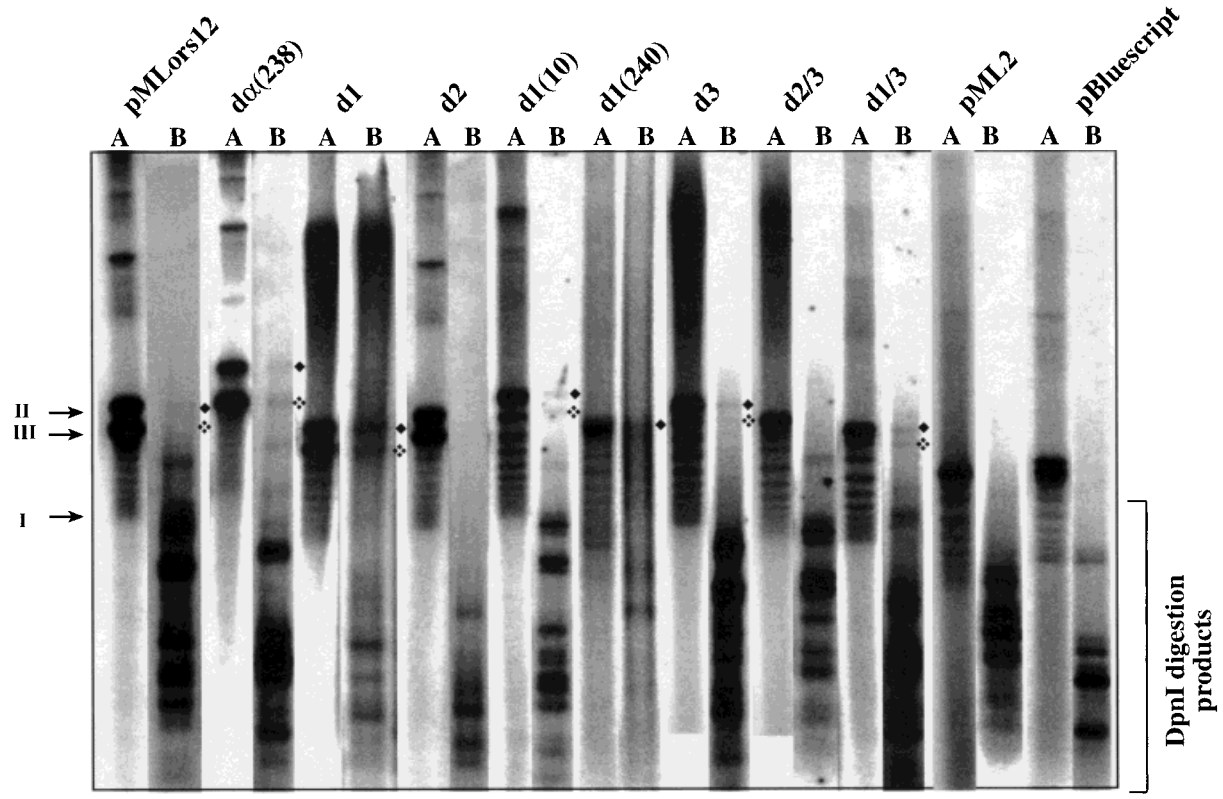

Fig. 4. In vitro replication of *ors12* deletion mutants. Reactions were performed as described in Materials and Methods. One fourth of each reaction was digested with *DpnI* (**B**), and subjected to electrophoresis on a 1% agarose gel overnight, together with one fourth of the undigested (**A**) replication products. The position of DNA forms I, II, and III (arrows), the *DpnI*-resistant products (form II, plain lozenges; form III, diamonds) and the *DpnI* digestion products (bracket), is indicated.

3D). Similarly, mutant dα(272), carrying a 272-bp deletion that removed both the α-satellite portion of *ors12* and the CACCC consensus (Fig. 1B), was not affected in its ability to replicate autonomously (data not shown). Overall, the results indicate that region 2 is sufficient to support the autonomous replication of *ors12* in HeLa cells, while region 1 may also contribute to this activity in the intact *ors12* (compare Fig. 3A and D to C).

In Vitro Replication of the Deletion Mutants

Each of the *ors12* deletion mutants was assayed for replication in vitro, in a system that uses HeLa cell extracts [Pearson et al., 1991]. The replication status of each mutant template DNA was verified by the *DpnI* resistance assay [Peden et al., 1980]. The same plasmid clones that exhibited autonomous replication activity in HeLa cells in vivo, by transfection (Figs. 2 and 3), were also capable of replicating in vitro. A comparison between the undigested (A) and the DpnI-digested (B) products of the in vitro replication reaction, revealed DpnI-resistant bands corresponding to forms II and III DNA for the plasmid containing the intact *ors12* sequence, as well as the deletion mutants dα(238), d1, d1(10), d1(240), d3 and d1/3 (Fig. 4 and Table I), indicating that those plasmids replicated in vitro. In contrast, the deletion mutants d2 and d2/3, in which region 2 was removed, did not produce any DpnI-resistant DNA (Fig. 4 and Table I). These results further substantiate the conclusions derived from the in vivo autonomous replication assays, and confirm that region 2 in *ors12*, comprised in the deletion mutant d1/3 (Fig. 1B), contains sequences important for its replication in vitro and in vivo.

Minimal Origin Sequence

The nucleotide sequence of region 2 (215-bp) was re-examined (Fig. 5), in light of its function as a putative minimal replication origin of *ors12*. The sequence is richer in G/C content (50.2%) than the full *ors12* sequence (47.5% G/C) and the 186-bp minimal origin of *ors8* (33.5% G/C)

TABLE I. Summary of the In Vivo and In Vitro Autonomous Replication Assays of *ors12* Deletion Mutants

	Replication status	
Deletion mutant	In vivo	In vitro
d1(10)	+	+
d1(240)	+	+
dα(238)	+	+
dα(272)	+	+
d1	+	+
d2	–	–
d3	+	+
d2,3	–	–
d1,3	+	+

[Todd et al., 1995]. The 215-bp fragment contains two of the IRs (22 and 16 bp long; ΔGs: −9 kcal and −12.3 kcal, respectively) [Rao et al., 1990] that are present in *ors12* (Figs. 1 and 5), which may assume a cruciform configuration in vivo [Zannis-Hadjopoulos et al., 1988, 1992], as well as multiple repeated sequence motifs. The most notable of these direct repeats are: CACACA, CTCTCT; TTTC, AGG, and CTTA (repeated 3 times each); and G(A)AG and TGGG(A) (repeated 4 times each) (Fig. 5).

DISCUSSION

ors12 (812 nucleotides in length) was previously isolated from African Green monkey kidney cells (CV-1), by nascent strand extrusion from replication bubbles activated at the onset of S-phase [Kaufmann et al., 1985], characterized [Zannis-Hadjopoulos et al., 1985] and sequenced [Rao et al., 1990]. It has been mapped to the centromere of a subset of six CV-1 chromosomes and a marker chromosome [Mah et al., 1992], and associates with the nuclear matrix in a cell cycle dependent manner [Mah et al., 1993]. It can function as an origin of semiconservative DNA replication in autonomously replicating plasmids in vivo [Frappier and Zannis-Hadjopoulos, 1987; Zannis-Hadjopoulos et al., 1994] and in a mammalian cell-free replication system [Pearson et al., 1991, 1994; Zannis-Hadjopoulos et al., 1992]. Recently we demonstrated, using competitive PCR mapping, that *ors12* functions as a chromosomal origin of replication in vivo, at its native locus (Pelletier et al., in preparation).

Here, we generated a panel of deletion mutants and tested them for autonomous replication function in vivo and in vitro, using the BrdUrd substitution assay and the *DpnI*-resistance assay, respectively. We found that an internal fragment (215-bp) of *ors12* is sufficient for its autonomous replication. This fragment, defined by the *ScaI* (nt 362) and *StyI* (nt 577) restriction sites (Fig. 1), is capable of autonomous replication when subcloned by itself in pML2, in the same replication assays. The results suggest that a minimal ori lies within the 215-bp (*ScaI-StyI*) fragment of *ors12*. This fragment comprises two IRs (22 and 16 bp long; ΔGs: −9 kcal and −12.3 kcal, respectively) and several directly repeated sequence motifs (Fig. 5). We have previously reported that origin-rich sequences (*ors*) isolated by nascent strand extrusion are enriched in IR (palindromic) sequences, which have the potential to form cruciform DNA [Zannis-Hadjopoulos et al., 1984; Pearson et al., 1996]. *ors12*, among other *ors*, showed evidence of active cruciform formation and involvement during DNA replication [Zannis-Hadjopoulos et al., 1992]. The presence of two IRs in the minimal origin region of *ors12* is consistent with those results. Using a similar analysis by deletion mutagenesis, Todd et al. [1995] showed that an 186-bp internal fragment of *ors8* constitutes the minimal origin of that *ors* and also comprises an IR (ΔG-7.4 Kcal), which gives rise to a cruciform structure in vivo and in vitro [Zannis-Hadjopoulos et al., 1988, 1992; Bell et al., 1991]. The 186-bp minimal functional origin of *ors8* also contains several directly repeated sequence motifs [Todd et al. 1995], but of a different nucleotide sequence than those found in *ors12* (this study). An *ors*-binding activity (OBA) capable of supporting the in vitro replication of *ors8* has been purified [Ruiz et al., 1995]. There is no significant nucleotide sequence homology (36% similarity index by the Wilbur/Lipmann DNA alignment over a region of 139-bp) between the 186-bp and the 215-bp minimal oris of *ors8* and *ors12*, respectively.

Region 1 of *ors12* (nucleotides 1–362) was also seemingly capable of contributing to its autonomous replication activity. Mutant d2/3 (Fig. 1B), which lacks region 1, although replication-negative in most experiments (Fig. 3B), was occasionally capable of replication (data not shown), suggesting that region 1 contains sequences that can sustain autonomous replication in vivo, in the absence of region 2. In the presence of region 2, however, region 1 is dispensable, as evidenced by the ability of deletion mutant d1 (Fig. 1B), lacking region 1 only, to

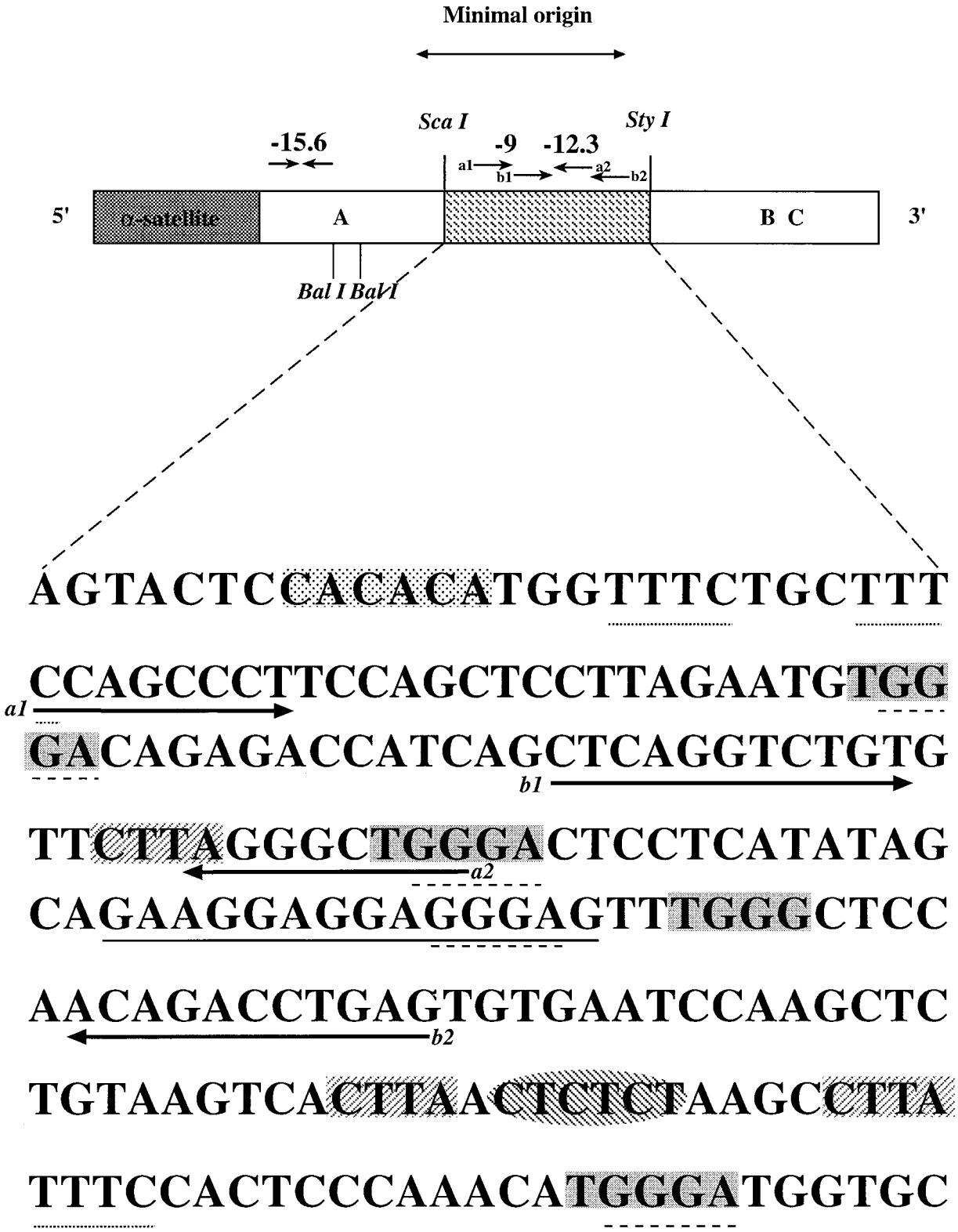

Fig. 5. Sequence detail of the 215-bp minimal origin region of *ors12* (deletion mutant d1–3). The repeated motifs are highlighted: CACACA (stippled box), CTCTCT (hatched ovoid), GA(A)G/GAG/GAG/(G)GAG (solid underline), CTTA (hatched boxes), TTTC (dotted underline), GGGA (dashed underline), and TGGG(A) (shaded boxes). The two inverted repeats are indicated (solid arrows; a1 pairs with a2 and b1 pairs with b2).

replicate efficiently (Fig. 2D). A similar observation has been reported in the yeast *Saccharomyces cerevisiae*, where the presence of the ABF-1 binding site enhances the replication of ARS plasmids, although its deletion does not abolish it [Marharens and Stillman, 1992]. Furthermore, in the yeast *Schizosaccharomyces pombe*, Clyne and Kelly [1995], using deletion and substitution analyses, found that ARS1 contains an essential core element and a large flanking domain that stimulates ARS activity. Finally, it was shown recently that replication initiation signals are redundant in the *c-myc* origin of replication [McWhinney et al., 1995].

Consensus sequences such as matrix (scaffold) attachment region (MAR or SAR) [Boulikas, 1992], pyrimidine tract [Yamaguchi et al., 1985], yeast ARS consensus (ACS) [Palzkill and Newlon, 1988], topoisomerase II binding site [Osheroff, 1989], or the Pur binding sequence [Bergemann et al., 1992] were not found in the 215-bp minimal origin. Similarly, no match was found to the consensus sequence (WAWTTDDWWW DHWGW HMAWTT) reported by Dobbs et al. [1994] as unique to potential initiation regions, nor to the asymmetric pyrimidine heptanucleotide consensus sequence (CTTTC(Py)(Py)) reported by Waltz et al. [1996]. The 3′-AT-rich region (nt 577–812) of *ors12*, which contains a sequence homologous (10/11-bp match) to the yeast ARS consensus (ACS) [Palzkill and Newlon, 1988] and a SAR-T consensus [Gasser and Laemmli, 1986; Rao et al., 1990], lie outside the 215-bp minimal ori. These results agree with those found previously for the 186-bp minimal ori of *ors8*, which also does not include these elements [Todd et al., 1995], although they are present in *ors8* [Rao et al., 1990]. Although seemingly not essential for *ors* function in the mammalian system, both the ACS and SAR-T are a common feature of the *ors* [Rao et al., 1990] and ACS is essential for ARS function in yeast [Van Houten and Newlon, 1990].

In summary, the results presented here suggest that a minimal ori lies within the 215-bp (*ScaI-StyI*) fragment of *ors12*. The importance of the various sequence motifs that are a feature of the 215-bp fragment is currently being investigated.

ACKNOWLEDGMENTS

We thank Asha Bahkar and Grant Auer for help with the construction of the deletion mutants. We also thank Veronica Klein for technical assistance in the in vitro replication assays.

REFERENCES

Bell D, Sabloff M, Zannis-Hadjopoulos M, Price G (1991): Anti-cruciform DNA affinity purification of active mammalian origins of replication. Biochim Biophys Acta 1089: 299–308.

Bergemann AD, Ma Z-W, Johnson EM (1992): Sequence of cDNA comprising the human pur gene and sequence-specific single-stranded-DNA-binding properties of the encoded protein. Mol Cell Biol 12:5673–5682.

Boulikas T (1992): Homeotic protein binding sites, origins of replication, and nuclear matrix anchorage sites share the ATTA and ATTTA motifs. J Cell Biochem 50:111–123.

Clyne RK, Kelly TJ (1995): Genetic analysis of an ARS element from the fission yeast schizosaccharomyces pombe. Embo J 14:6348–6357.

Dierks P, Van Doyen A, Cochran MD, Dobkin C, Reiser J (1983): Three regions upstream from the cap site are required for efficient and accurate transcription of the rabbit beta-globin gene in mouse 3T3 cells. Cell 32:695–706.

Dobbs DL, Shaiu W-L, Benbow RM (1994): Modular sequence elements associated with origin regions in eukaryotic chromosomal DNA. Nucleic Acids Res 22:2479–2489.

Frappier L, Zannis-Hadjopoulos M (1987): Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. Proc Natl Acad Sci USA 84:6668–6672.

Gasser SM, Laemmli UK (1986): Cohabitation of scaffold binding proteins with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. Cell 46:521–530.

Graham FL, Van Der Eb AJ (1973): A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.

Hirt B (1967): Selective extraction of polyoma DNA from infected mouse cell cultures. J Mol Biol 26:365–369.

Kaufmann G, Zannis-Hadjopoulos M, Martin RG (1985): Cloning of nascent monkey DNA synthesized early in the cell cycle. Mol Cell Biol 5:721–727.

Landry S, Zannis-Hadjopoulos M (1991): Classes of autonomously replicating sequences are found among early-replicating monkey DNA. Biochim Biophys Acta 1088: 234–244.

Mah DCW, Shihab-El-Deen A, Price GB, Zannis-Hadjopoulos M (1992): *ors12*, a mammalian autonomously replicating DNA sequence, is present at the centromere of CV-1 cell chromosomes. Exp Cell Res 203:435–442.

Mah DCW, Dijkwel PA, Todd A, Klein V, Price GB, Zannis-Hadjopoulos M (1993): *ors12*, a mammalian autonomously replicating DNA sequence, associates with the nuclear matrix in a cell cycle dependant manner. J Cell Sci 105:807–818.

Maniatis T, Fritsch EF, Sambrook J (1989): "Molecular Cloning: A Laboratory Manual." 2nd ed. New York: Cold Spring Harbor Laboratory.

Marahrens Y, Stillman B (1992): A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science 255:817–823.

McWhinney C, Waltz SE, Leffak M (1995): *Cis*-acting effects of sequences within 2.4-kb upstream of the human *c-myc* gene on autonomous plasmid replication in HeLa cells. DNA Cell Biol 14:565–579.

Osheroff N (1989): Biochemical basis for the interactions of type I and type II topoisomerases with DNA. Pharmacol Ther 41:223–241.

Palzkill TG, Newlon CS (1988): A yeast replication origin consists of multiple copies of a small conserved sequence. Cell 53:441–450.

Pearson CE, Frappier L, Zannis-Hadjopoulos M (1991): Plasmids bearing mammalian DNA-replication origin-enriched (ors) fragments initiate semiconservative replication in a cell-free system. Biochim Biophys Acta 1090: 156–166.

Pearson CE, Shihab-El-Deen A, Price GB, Zannis-Hadjopoulos M (1994): Electron microscopic analysis of in vitro replication products of *ors8*, a mammalian origin enriched sequence. Somat Cell Mol Genet 20:147–152.

Pearson CE, Zorbas H, Price GB, Zannis-Hadjopoulos M (1996): Inverted repeats, stem-loops, and cruciforms: significance for initiation of DNA replication. J Cell Biochem 63:1–22.

Peden KWC, Pipas JM, Pearson-White S, Nathans D (1980): Isolation of mutants of an animal virus in bacteria. Science 209:1392–1396.

Rao BS, Zannis-Hadjopoulos M, Price GB, Reitman M, Martin RG (1990): Sequence similarities among monkey ori-enriched (*ors*) fragments. Gene 87:233–242.

Ruiz MT, Pearson CE, Nielsen T, Price GB, Zannis-Hadjopoulos M (1995): Cofractionation of hela cell replication proteins with ors-binding activity. J Cell Biochem 58:221–236.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Todd A, Landry S, Pearson CE, Khoury V, Zannis-Hadjopoulos M (1995): Deletion analysis of minimal sequence requirements for autonomous replication of *ors8*, a monkey early-replicating DNA sequence. J Cell Biochem 57: 280–289.

Van Houten JV, Newlon CS (1990): Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol Cell Biol 10:3917–3925.

Waltz SE, Trivedi AA, Leffak M (1996): DNA replication initiates non-randomly at multiple sites near the c-myc gene in HeLa cells. Nucleic Acids Res 10:1887–1894.

Wu C, Friedlander P, Lamoureux C, Zannis-Hadjopoulos M, Price GB (1993): cDNA clones contain autonomous replication activity. Biochim Biophys Acta 1174:241–257.

Yamaguchi M, Hendrickson EA, DePamphilis M (1985): DNA primase-DNA polymerase alpha from simian cells. Modulation of RNA primer synthesis by ribonucleoside triphosphates. J Biol Chem 260:6254–6263.

Zannis-Hadjopoulos M, Persico M, Martin RG (1981): The remarkable instability of replication loops provides a general method for the isolation of origins of DNA replication. Cell 27:155–163.

Zannis-Hadjopoulos M, Kaufmann G, Martin RG (1984): Mammalian DNA enriched for replication origins is enriched for snap-back sequences. J Mol Biol 179:577–586.

Zannis-Hadjopoulos M, Kaufmann G, Wang S-S, Lechner RL, Karawya E, Hesse J, Martin RG (1985): Properties of some monkey DNA sequences obtained by a procedure that enriches for DNA replication origins. Mol Cell Biol 5:1621–1629.

Zannis-Hadjopoulos M, Frappier L, Khoury M, Price GB (1988): Effect of anti-cruciform DNA monoclonal antibodies on DNA replication. EMBO J 7:1837–1844.

Zannis-Hadjopoulos M, Pearson CE, Bell D, Mah D, McAlear M, Price GB (1992): Structural and functional characteristics of autonomously replicating mammalian origin-enriched sequences (ORS): In: Hughes P, Fanning E, Kohiyama M (eds): "DNA Replication: The Regulatory Mechanisms." Berlin: Springer-Verlag, pp 107–116.

Zannis-Hadjopoulos M, Nielsen TO, Todd A, Price GB (1994): Autonomous replication in vivo and in vitro of clones spanning the region of the DHFR origin of bidirectional replication (*ori*β). Gene 151:273–277.