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Functional Genomic Mapping of an Early-Activated Centromeric Mammalian Origin of DNA Replication

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Abstract *Ors12,* a mammalian autonomously replicating sequence (812 bp), was previously isolated by extrusion of African green monkey (CV-1 cells) nascent DNA from active replication bubbles. It contains a region of α-satellite extending 168-bp from the 5′-end, and a nonrepetitive portion extending from nucleotide position 169 to nucleotide 812 that is present in less than nine copies per haploid genome. *Ors12* is capable of transient autonomous DNA replication in vivo and in vitro, associates with the nuclear matrix in a cell cycle-dependent manner, and hybridizes at the centromeric region of six CV-1 cell chromosomes as well as a marker chromosome. To demonstrate that DNA replication initiates at *ors12* at a native chromosomal locus, a 14.2 kb African green monkey genomic clone was isolated and sequence information was obtained that allowed us to generate eight sets of PCR primers spanning a region of 8 kb containing *ors12.* One set of primers occurred inside *ors12.* These primers were used to amplify nascent DNA strands from asynchronously growing CV-1 and African green monkey kidney (AGMK) cells, using noncompetitive and competitive PCR-based mapping methodologies. Both assays showed that DNA replication in vivo initiates preferentially in a 2.3 kb region containing *ors12,* as well as at a second site located 1.7 kb upstream of *ors12.* This study provides the first demonstration of genomic function for a centromeric mammalian origin of DNA replication, originally isolated by nascent strand extrusion. J. Cell. Biochem. 74:562–575, 1999. © 1999 Wiley-Liss, Inc.

Key words: origin; DNA replication; competitive PCR; mapping; autonomous replication

Replication origins, or replicators, are the cis-acting sequences that direct the initiation of DNA synthesis [DePamphilis, 1993; Stillman, 1993; Diller and Raghuraman, 1994]. Regulation of DNA replication occurs primarily at the level of initiation [Hand, 1978; Kornberg and Baker, 1992; Gavin et al., 1995]. Although a great deal of information has been obtained from studies with the budding yeast *S. cerevisiae* [reviewed in Brewer and Fangman, 1991], studies of replication origins in mammalian cells have advanced more slowly. Yeast replicators [Stillman, 1993] were identified through their ability to confer high autonomous replication potential (ARS function) on plasmids [Chan and Tye, 1980; Stinchcomb et al., 1980]. Most ARS have been shown to act as replication start sites on the yeast chromosomes [Dubey et al., 1991].

Different strategies have been adopted for isolating origin sequences in mammalian cells [Vassilev and DePamphilis, 1992; DePamphilis, 1993]. The origins controlling the replication of amplified regions of the DHFR gene in Chinese hamster cells [Burhans et al., 1986; Vaughn et al., 1990], the CAD gene in Syrian hamster cells [Carroll et al., 1987] and the adenosine deaminase (ADA) gene in mouse cells [Carroll et al., 1993] were studied either at their respective amplicons or at their single-copy loci in the original nonamplified cell lines [Carroll et al., 1987; Dijkwel and Hamlin, 1995; Kelly et al., 1995]. Our laboratory has isolated origin-enriched sequences (*ors*) from African green monkey kidney (CV-1) cells by extrusion of nascent DNA [Zannis-Hadjopoulos et al., 1981] from small replication bubbles at the onset of S-phase [Kaufmann et al., 1985]. Many studies have relied on transient autonomous replication assays to demonstrate origin activity of sequences such as those upstream of the *c-myc* gene [Iguchi-Ariga et al., 1988; McWhin-

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ney et al., 1995], in a library of *ors* fragments [Frappier and Zannis-Hadjopoulos, 1987; Wu et al., 1993a], in random human fragments [Krysan et al., 1989], in double-minute chromosomes containing the ADA locus [Virta-Pearlman et al., 1993], in the promoter of the human *hsp70* gene [Taira et al., 1994] and in the murine immunoglobin enhancer [Iguchi-Ariga et al., 1993], among many others. In addition, a number of methods have been developed for the physical mapping of DNA replication initiation sites (*ori*) [reviewed in Vassilev and DePamphilis, 1992]. These methods include: analysis of the replication intermediates on 2D-gels [Brewer and Fangman, 1987; Huberman et al., 1987], amplification of nascent DNA strands by PCR [Vassilev and Johnson, 1989, 1990; Wu et al., 1993b], determination of replication fork polarity by analysis of the synthesis of Okazaki fragments along the zone of initiation [Burhans et al., 1990] or analysis of leading strand asymmetry in cells inhibited for Okazaki fragment synthesis [Handeli et al., 1989], identification of the earliest labeled fragments [Gale et al., 1992], and determination of nascent DNA strand abundance by competitive PCR [Giacca et al., 1994; Kumar et al., 1996; Pelizon et al., 1996] or by hybridization with defined origin sequences [Yoon et al., 1995].

Ors sequences are capable of transient autonomous replication in vivo, upon transfection in monkey (CV-1 and Cos-7) and human (HeLa) cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Wu et al., 1993a], and in an in vitro replication system using HeLa cell extracts [Pearson et al., 1991]. Both in vivo and in vitro replication initiates within the *ors,* is semi-conservative, bidirectional, and sensitive to the action of aphidicolin [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991, 1994]. Among the functional *ors, ors12* has been analyzed in detail both in vivo [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Zannis-Hadjopoulos et al., 1994] and in vitro [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1992, 1994]. Recently, we identified by deletion analysis a 215-bp internal fragment as the minimal sequence required for origin function of *ors12* in vivo and in vitro [Pelletier et al., 1997]. A sequence homologous to *ors12* is present in less than nine copies per haploid genome of CV-1 cells and was localized by in situ hybridization to the centromere of six CV-1 chromosomes and that of a marker chromosome [Mah et al., 1992]. Restriction mapping analysis of CV-1 genomic DNA revealed a high degree of conservation in the arrangement of the different *ors12* copies (Mah et al., 1992]. *Ors12* associates with the nuclear matrix in a cell cycle-dependent manner and replicates on the matrix in early- and mid-S phase [Mah et al., 1993]. It contains 168 bp of α-satellite repetitive DNA at its 5′-end, while near its 3′-end, among other features, it contains an imperfect match (10/11 bp) to the ARS consensus of yeast, AT-rich regions and a scaffold attachment consensus (SAR-T) of Drosophila [Rao et al., 1990].

In this study, using both noncompetitive and competitive PCR-based origin-mapping methodologies, we show that *ors12* serves as a DNA replication initiation site at its chromosomal locus in the African green monkey kidney cell line, CV-1, and in primary (AGMK) cells. We also show the existence of a second site of initiation located approximately 1.7 kb upstream of *ors12.*

MATERIALS AND METHODS

Cell Cultures

CV-1 cells (monolayers) were cultured in Dulbecco's minimal Essential medium (DMEM) supplemented with 10% fetal calf serum, as previously described [Kaufmann et al., 1985]. African green monkey kidney (AGMK) cells (Biowhittaker, Walkersville, MD) were cultured in MEMα medium supplemented with 10% fetal bovine serum (FCS, Flow Lab., McLean, VA) as previously described [Mah et al., 1992]. For the noncompetitive PCR mapping experiments, exponentially growing cells were pulse-labeled for 15 min with bromodeoxyuridine (BrdU, 20 μM) and nascent DNA was prepared as described before [Vassilev and Johnson, 1990; Wu et al., 1993b]. For synchronization to the G0 phase, AGMK cells were put in serum-free medium for 48 h, and the position in the cell cycle was verified by FACS analysis.

Genomic Clone Isolation

A λ Charron 40A genomic library (Mk2-Ch40A, a generous gift from Dr. A. Maresca, University Laval, Québec) made from African green monkey liver genomic DNA digested with Mbo1 (fragments of 9 to 23 kb) was screened using Zeta-probe (Bio-Rad, Richmond, VA) membranes following the manufacturer's recommen-

dations. The plaque lifts were hybridized with the nonrepetitive portion of *ors12* (215-bp ScaI-StyI fragment [Mah et al., 1992]). A phage recombinant clone containing an insert of 14.2 kb was obtained, mapped with restriction enzymes and subcloned into pBluescript KS(+) plasmid propagated in DH5αF′ bacterial strain, according to standard procedures [Maniatis et al., 1989]. The 5′- and 3′-ends of some of the subclones were sequenced by the dideoxynucleotide chain termination method [Sanger et al., 1977].

Purification of Nascent DNA

AGMK or CV-1 cells at 60–80% confluency were lysed by addition of 4 ml of lysis buffer (0.5% SDS, 1M NaCl, 0.5M EDTA, and 1M Tris-HCl, pH 8.0) per flask (175 cm^2) and total DNA was recovered as previously described [Maniatis et al., 1989]. The DNA was then denatured with NaOH (0.2N final concentration) and equal amounts were then layered on three 5% to 30% denaturing (NaOH 0.2N, EDTA 3 mM) sucrose gradients. A fourth identically prepared sucrose gradient was loaded with molecular weight markers (λ/HindIII-EcoRI and pBluescript KS(−)/HaeIII). All gradients were centrifuged in a SW27 rotor at 15°C for 20 h at 26 krpm. Fractions of 1.5 ml were collected from the top, as described before [Wu et al., 1993a]. The nascent DNA was precipitated in ethanol, using linear polyacrylamide (20 μg/ml) as carrier, and the pellets were resuspended in 400 μl TE.

Oligonucleotide Primers and Competitors

Eight sets of PCR primers were generated by chemical synthesis in selected nonrepetitive segments of the sequence obtained from the genomic clone (Fig. 1). The name and approximate distance of each amplified segment from the 5′ end of the genomic insert is as follows: K-K′: 660 bp; A-A′: 850 bp; B-B′: 1,450 bp; J-J′: 2,020 bp; D-D′: 2,500 bp; M-M′: 4,270 bp; C-C′: 6,750 bp; E-E′: 7,600 bp. A control set of primers was generated in the exon 11 of African green monkey BRCA2 gene [Bignell et al., 1997] (GeneBank accession #Z75666). The location of each primer in the sequence is as follows: BRCA2 forward (F): 2761-2780; BRCA2 reverse

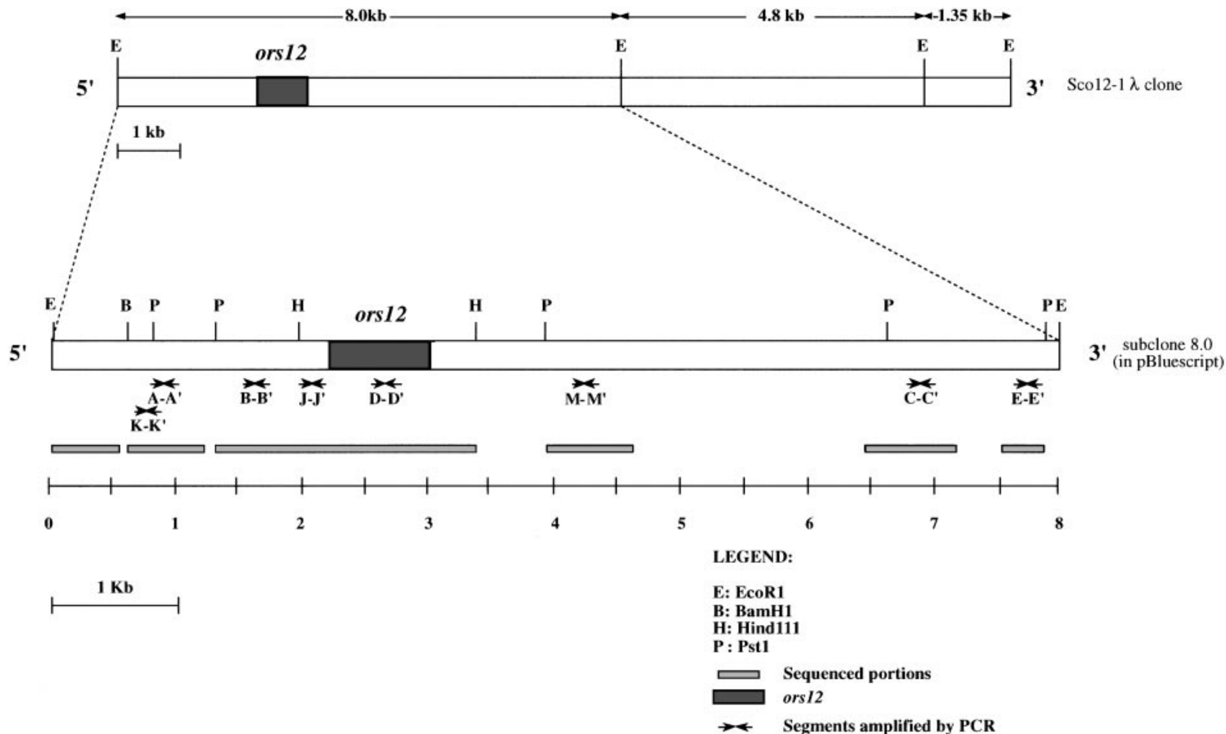

Fig. 1. Schematic representation of the genomic region under study. The top part shows the restriction map of a recombinant clone containing *ors12* as isolated from an African green monkey genomic library. The bottom part presents the restriction map of the 8.0 kb EcoR1 fragment subcloned into pBluescript-KS(+) plasmid. The portions that were sequenced are indicated by hatched bars. The positions of the PCR primers used for the mapping experiments (Figs. 2 through 5) are depicted by the inverted arrows underneath the map.

(R): 3219-3200. The length and sequence of the segments amplified by each pair of primers is shown in Table I.

For each set of primers synthesized, a specific competitor was constructed. For primer sets A-A′, B-B′, C-C′, and D-D′, this was accomplished by selectively deleting a segment of DNA from the full-length PCR product using restriction enzymes; the digestion products were resolved on a 6% polyacrylamide gel, and the two end-fragments (containing the primer target sequences) were recovered by isotachophoresis [Ofverstedt et al., 1984] and religated together using T4 DNA ligase (New England Biolabs, Beverly, MA). This DNA was subjected to a new round of PCR amplification using the original primers, and the products were resolved on a 6% polyacrylamide gel, as described above. The band corresponding to the competitor was touched with the tip of a needle, which was then soaked in a tube containing fresh PCR reagents and the DNA was reamplified. The amplification product was then subjected to electrophoresis on a 6% polyacrylamide gel, as described above, eluted by the crush and soak method [Maniatis et al., 1989] and quantified. For primer set E-E′, a competitor was constructed by amplifying CV-1 genomic DNA with low stringency primer annealing conditions, as described by Förster [1994b]. For the remaining sets of primers (K-K′, J-J′, M-M′, and BRCA2 F-BRCA2 R), a competitor was constructed using a third primer, as described by Förster [1994a]. The length of each of the competitor products as well as the sequence of the primers used for the construction of the competitors (“comp”) is given in Table I. The amount of competitor DNA was determined by spectrophotometry (Beckman DU-65, Beckman Instruments, Fullerton, CA). For verification, a constant amount of each competitor (except the BRCA2 comp) was challenged with increasing amounts of a plasmid clone of known concentration carrying the target sequences of all primer sets (subclone 8.0, Fig. 1). The products were resolved on a 2% agarose gel, stained with ethidium bromide, and photographed with an Eagle Eye apparatus (Speed Light/BT Scientech-LT1000). The picture was quantitated by densitometry with a Bio Image analyser (MilliGen/Bioresearch, Division of Millipore, Bedford, MA). Multiple exposures (Eagle Eye) were performed to obtain band intensities within the linear range of the densitometer. The plasmid/

TABLE I. Oligonucleotides and PCR Conditions Used[a]

Primer	Sequence	Product size
A	5′CCCAGCCTATTCTCTCTTTA3′	Genomic: 282 bp
A′	5′CCCTAGTCTAAGCCCTCAGT3′	Competitor: 263 bp
K	5′GAGACAAGGCTGTCCCTCAG3′	Genomic: 394 bp
K′	5′TAGCATTTCATGAGCATCCG3′	Competitor: 364 bp
K comp	5′TGAGCATCCGGTCATCCCCATCTGGGCAGT3′	
B	5′CCCTTGATTAATGGTTGCTT3′	Genomic: 463 bp
B′	5′GCTGGTGGGGAATGTTAATG3′	Competitor: 400 bp
J	5′CAGACATCAGCAAGTGACGG3′	Genomic: 360 bp
J′	5′TAGCCAATGTGCCCAATGTA3′	Competitor: 330 bp
J comp	5′GCCCAATGTAAGAGCCCTGGTACATGTGTG3′	
D	5′TTCAGGACCATACAGCATGG3′	Genomic: 374 bp
D′	5′ACATGAGATACAGGCAGTGC3′	Competitor: 319 bp
M	5′CATTCGTTCATCCATGTCTCC3′	Genomic: 303 bp
M′	5′GTGAATGAGGCAGTTTGAGGA3′	Competitor: 273 bp
M comp	5′AGTTTGAGGAATTGCGGAAAAGAGACAGAG3′	
C	5′TTGAGGGAAAGTAGGCATCC3′	Genomic: 343 bp
C′	5′AGTTTTACTTAGCCCCTGAC3′	Competitor: 301 bp
E	5′GGAATTCTGTCTTAGGCAAT3′	Genomic: 250 bp
E′	5′TGATATTGCCAATCAGGATC3′	Competitor: 195 bp
BRCA2 F	5′GATCACAACTGCCCCAAAGT3′	Genomic: 459 bp
BRCA2 R	5′TCTTGTTTTTCGGAGGGATG3′	Competitor: 429 bp
BRCA2 comp	5′CGGAGGGATGTGAATTATTTTCATACAAAG3′	

[a]The PCR profile used for each set of primers is given in Materials and Methods. The size of the PCR products obtained with genomic DNA (“genomic product”) and the size of each of the competitor products (“competitor product”) is also indicated. The oligonucleotides used for the construction of some of the competitors are labelled “comp.”

competitor ratio was plotted against the amount of plasmid added, and the concentration of the competitor was calculated from the amount of plasmid interpolating at 1:1 ratio.

PCR Conditions

For the conventional (semi-quantitative) PCR mapping, 5 μl (one-tenth volume) of each size fraction were used in a PCR reaction with primer sets A-A′, C-C′, D-D′, and E-E′ with 2.5 units Taq polymerase (Pharmacia, Gaithersburg, MD) in a Perkin-Elmer Cetus DNA thermal cycler 480, using 30 cycles of the profiles that are shown below. Elongation at 72°C was performed for 5 min at the end of the last cycle. The products were analyzed on a 6% PAGE gel and stained with ethidium bromide. For the competitive PCR experiments, 2 μl of nascent DNA from each fraction (400 μl) were coamplified with each of the competitors, as described by Pelizon et al. [1996], with 1 unit Taq polymerase (Pharmacia) in a Perkin-Elmer Cetus DNA thermal cycler 480, using 30 cycles of the following profiles: 94°C, 60°C, 72°C (for primer sets K-K′, B-B′, J-J′, D-D′, and M-M′), or 94°C, 55°C, 72°C (for primer sets A-A′, C-C′, and E-E′). Each step was performed for 30 sec. Again, a 5-min elongation step at 72°C was performed at the end of the last cycle. The products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and analyzed.

RESULTS

Semi-Quantitative PCR Mapping of *ors12*

In order to demonstrate the function of *ors12* as a replication origin at its chromosomal locus in CV-1 cells, the PCR-based method for mapping initiation sites of DNA replication in vivo [Vassilev and Johnson, 1989] was employed, as modified by Virta-Pearlman et al. [1993]. In this method, exponentially growing cells are pulse-labeled for 15 minutes with bromodeoxyuridine (BrdU), and total DNA is extracted. The nascent DNA is separated from the parental DNA by sedimentation through alkaline cesium gradients, recovered from the corresponding heavy fractions of the gradient, size-fractionated on an alkaline sucrose gradient and precipitated. The DNA from each fraction is subsequently amplified by PCR with different sets of primers spanning the region containing the putative origin of DNA replication. The shortest nascent strands are amplified only by the primers located at or close to the origin, while the longer strands are amplified by the primers located further away from it, either upstream or downstream.

To apply this method to *ors12,* a 14.2 kb genomic clone containing *ors12* was isolated from a genomic library of African green monkey, using the nonrepetitive portion (215-bp ScaI-StyI fragment) of *ors12* as probe [Mah et al., 1992], selected regions were sequenced, and suitable PCR primers were generated (Fig. 1 and Table I), as described in Materials and Methods.

Nascent DNA from asynchronously growing CV-1 cells was isolated and fractionated as described above. An aliquot of each size-fraction was subjected to electrophoresis on agarose gel, and then blot-transfered and hybridized with ^{32}P-labeled total genomic DNA for verification of its length and the quality of the DNA (Fig. 2A). Each fraction contained strands of increasing length ranging in size from a few hundred nucleotides (nt; Fig. 2A, lane 2) to approximately 15,000 nt (Fig. 2A, lane 6) corresponding to nascent strand lengths previously reported by others [Ariizumi and Tucker, 1993]. An aliquot of the DNA from each fraction was then amplified by PCR using primer sets A-A′, D-D′, and C-C′, respectively (Table I). The three sets of primers were included simultaneously in the same tube, to minimize the tube to tube variability often arising from the PCR process [Diviacco et al., 1992]. The results (Fig. 2B) show that the sucrose gradient fraction #3 contained nascent strands of the minimum length necessary to include segments A-A′, and D-D′ (Fig. 2B, lane A), while sucrose gradient fraction #6, containing the longest nascent DNA strands tested, was required to amplify the segments C-C′ and E-E′ (Fig. 2B, lanes D and F). Assuming that replication is bidirectional, originates from *ors12,* and that the two forks move at a similar rate, then only nascent strands of a size greater than 8,500 nt should be amplified by the primer sets C-C′ and E-E′, since fraction #6 alone contains nascent DNA molecules of sufficient length to be amplified by these primers. No products were obtained from fraction #2 (not shown), indicating that the nascent DNA chains present in that fraction, were either underrepresented or too short to be amplified by any of the primer sets. Overall, these results suggested that both primer sets

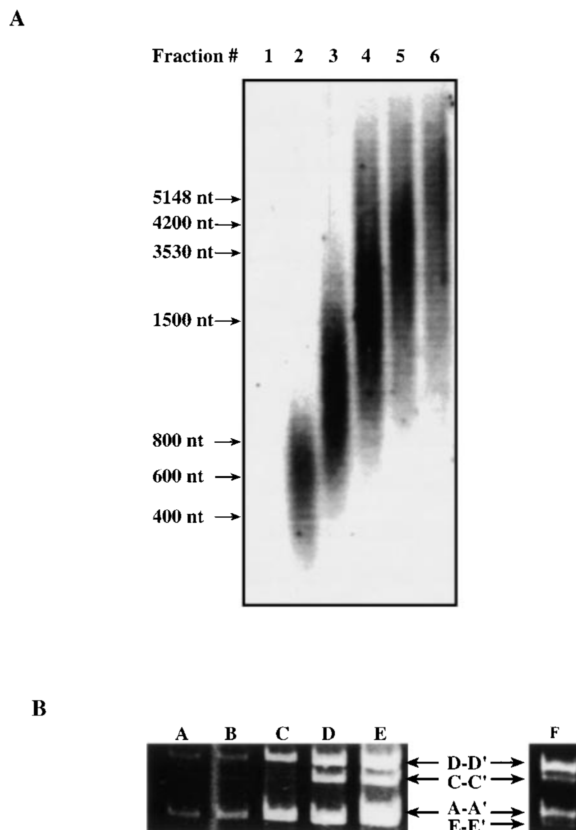

Fig. 2. Semi-quantitative PCR mapping analysis of *ors12* in CV-1 cells. **A:** Newly synthesized, BrdU-labelled DNA isolated from CV-1 log phase cells was size fractionated on a linear sucrose gradient and the fractions collected from the top were precipitated. An aliquot of each was run on a 1% alkaline agarose gel, blotted and hybridized with ^{32}P-labelled CV-1 chromosomal DNA. The DNA markers are λ/HindIII-EcoRI and 100-bp ladder (GIBCO-BRL). The average size of the nascent DNA was determined by comparison with the markers. **B:** Newly synthesized DNA from the sucrose fractions was amplified with the primer sets A-A′, D-D′, and C-C′ simultaneously, and the products for each fraction were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. **Lanes A, B, C,** and **D** represent the products obtained with fractions 3, 4, 5, and 6, respectively, from the sucrose gradient. The products in **lane E** were obtained from amplification of 100 ng of CV-1 genomic DNA with the same primers. **Lane F** represents the products obtained by amplifying fraction #6 with primers A-A′, D-D′, C-C′, and E-E′, simultaneously, which were resolved on a 6% polyacrylamide gel.

A-A′ and D-D′ were able to amplify the shortest nascent strands and, thus, must lie closest to an origin of DNA replication, while primers C-C′ and E-E′ must lie further away from it.

The specificity of the primers was tested using total CV-1 genomic DNA as template in the PCR reaction. After 30 cycles of amplification of 100 ng of DNA, the reaction products were subjected to electrophoresis on an agarose gel and analyzed as above. Each pair of primers generated a distinct single band corresponding in size to the length of DNA between the respective pairs of primers (Fig. 2B, lane E).

Competitive PCR Mapping of *ors12*

An alternative method was developed recently for the mapping of origins of DNA replication that combines the sensitivity of the conventional PCR technique for mapping genomic origins of replication [Vassilev and Johnson, 1989] with the quantitative reproducibility of competitive PCR [Gilliland et al., 1990; Diviacco et al., 1992; Zimmermann and Mannhalter, 1996]. By coamplification of a constant amount of nascent DNA with increasing amounts of a competitor that shares the same primer target sites, but differs slightly in length from the genomic product, the abundance of nascent DNA molecules synthesized at the replication origin and at sites flanking it can be measured, enabling the precise localization of the site of initiation. This method was used to map the origins associated with the human lamin B2 [Giacca et al., 1994] and the CHO DHFR [Pelizon et al., 1996; Kobayashi et al., 1998] genes as well as for demonstrating differential origin activities in human normal skin fibroblasts and HeLa cell lines [Tao et al., 1997].

To confirm the results obtained by the conventional PCR method (see above), we applied competitive PCR to the *ors12* locus. In order to analyze the region in more detail and to map the initiation zone more accurately, additional primer sets (K-K′, B-B′, J-J′, and M-M′) were generated. A series of competitor templates was then constructed for each set of primers; each competitor contains the same primer target sequences at its extremities, but is shorter by a few (30–60) nucleotides than its equivalent genomic product (Table I). All products were coamplified with a plasmid clone (Sco12-8.0), whose genomic insert (8.0 kb EcoRI fragment, Fig. 1) comprises the target sites of all the primers, and were quantitated in relation to it, thus avoiding variability in quantification. In order to test the relative amplification efficiency of the primers, each set was first used in quantitative PCR amplification of their target sequence in genomic DNA. This was accomplished by quantitating the number of parental DNA chains found in AGMK genomic (non-nascent) DNA, in cells that were synchronized to the G0 phase by serum starvation for 48 h. After syn-

chronization, total genomic DNA was extracted and used for competitive PCR amplification with each set of primers (Fig. 1 and Table I). All primer sets amplified their corresponding target sequence in genomic DNA with similar efficiencies (approx. 120–200 molecules per ng of DNA; Fig. 3), with the exception of primer sets K-K′, which had the lowest efficiency of amplification (approx. 50 molecules per ng of DNA) and those of an unrelated control sequence, the monkey BRCA2 gene (51; see below), which had the highest (approx. 270 molecules per ng of DNA).

For the origin mapping experiments, nascent DNA was isolated from asynchronously growing primary AGMK cells and CV-1 cells, and was fractionated by size on an alkaline sucrose gradient. Gradient fractions containing small (<1000 nt) DNA strands should be highly enriched for nascent DNA and should not contain any significant amount of parental DNA [Kumar et al., 1996; Tao et al., 1997]. For the AGMK cells, gradient fractions were selected, with DNA strands ranging in size from approximately 250–950 nt (approximate average size of 600 nt) and 770–1,600 nt (approximate average size of 1,200 nt), respectively, as determined by a separate gradient containing appropriate molecular weight markers and by alkaline agarose gel electrophoresis (Fig. 4). For the CV-1 cells, two equivalent fractions were selected, with DNA strands ranging in size from approximately 175–770 nt (approximate average size of 500 nt) and 750–1,600 nt (approximate average size of 1,200 nt), respectively (Fig. 4). This nascent DNA was used to perform competitive PCR amplification with each set of primers and their corresponding competitor and the products were analyzed by electrophoresis. The intensity of each band generated by each primer set was quantitated by densitometry, the ratio of competitor product to target (nascent DNA) product was calculated, and the results were plotted against the amount of competitor added to each reaction. Typical examples of such plots, obtained with the AGMK nascent DNA from the fraction ranging in size between 250–950 nt, are shown in Figure 5. For each pair of primers, a linear relationship was observed with a regression coefficient and a slope of approximately 1 (slope ranging between 0.74 and 1.28, and regression coefficient ranging between 0.942 and 0.996), indicative of equal competitiveness of the target and the competitor DNAs for the primers and other reagents in the reaction, in accordance to the rules of competitive PCR [Gilliland et al., 1990; Raemaekers, 1995]. The number of nascent DNA molecules present in each fraction of the gradient was determined by finding the number of competitor molecules required for a ratio of competitor to target equal to 1.

The results were corrected for the relative copy number of each primer set, by dividing the number of nascent strands found at each amplified segment by the abundance of the same segment in AGMK parental DNA, as determined above (see Fig. 3). The corrected results were plotted as a percentage of the relative abundance of nascent strands for each primer set used (Fig. 6). For the AGMK fraction, averaging approx. 600 nt, the majority of nascent

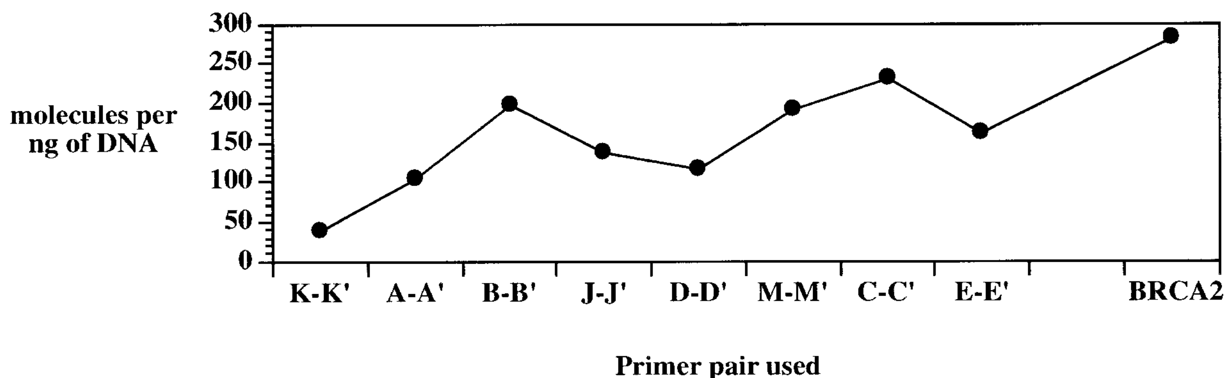

Fig. 3. Quantitative amplification of control AGMK parental DNA. African green monkey primary kidney cells were arrested in G0 by serum starvation for 48 h, and the genomic (parental) DNA was extracted as previously described (Materials and Methods). An aliquot of this DNA (2 μl out of 1.5 ml, corresponding to 185 ng of DNA) was then used in control competitive PCR amplifications with each set of primers. The results (line) represent the relative abundance of parental DNA strands found at each PCR target sequence, expressed per ng of DNA. The quantification was done as in explained in Figure 5.

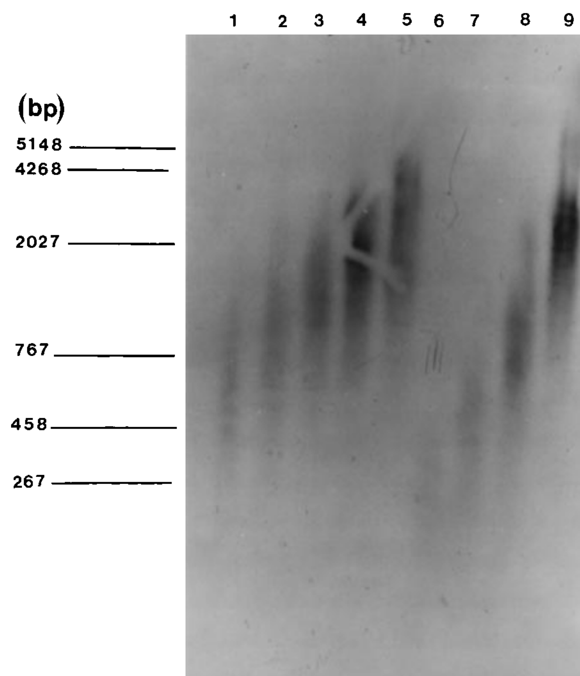

Fig. 4. Size fractionation of nascent AGMK and CV-1 DNA. Newly synthesized DNA isolated from log phase CV-1 (**lanes 1–5**) and AGMK (**lanes 6–9**) cells was size fractionated on an alkaline agarose gel (1.5%). The DNA markers were generated by digesting λ DNA with HindIII and EcoRI. The average size of the nascent DNA was determined by comparison with the markers. Lanes 1–5 represent the products obtained with fractions 6–10, respectively, from the sucrose gradient of CV-1 nascent DNA; lanes 6–9 represent the products obtained with fractions 5–9, respectively, from the sucrose gradient of AGMK nascent DNA.

strands was amplified by primer sets A-A′, which is located approximately 1.7 kb upstream of *ors12* (Fig. 6A, A-A′, black bars) and D-D′, corresponding to the *ors12* site (Fig. 6A, D-D′, black bars). This portion of nascent strands was enriched more than 10-fold by comparison to the nascent strands amplified by primer sets M-M′, C-C′, and E-E′, which are located on the 3′ side of *ors12,* at a distance of 1.8 kb, 4.3 kb, and 5.1 kb, respectively, approximately three- to four-fold by comparison to the nascent strands amplified by primer sets B-B′ and J-J′, located within 1.1 Kb upstream of *ors12,* between the A-A′ and *ors12* sites, and approximately 1.3-fold by comparison to the nascent strands amplified by primer set K-K′, located approximately 300 bp upstream of the A-A′ site. The results are consistent with a bidirectional replication fork movement from the two independent origin activities observed at the *ors12* and A-A′ sites, respectively.

A similar pattern was obtained with DNA from the fraction containing longer (approx. 1,200 nt) nascent strands (Fig. 6A, stippled bars). An increased abundance of molecules was found at the A-A′ (approx. 14,000 molecules) and D-D′ (approx. 12,000 molecules) segments, relative to those found at the other segments (approx. 1,000–4,000 molecules). From the distance between the DNA segments amplified by primers B-B′, J-J′, and D-D′ as well as by the distribution of the abundance of nascent strands obtained at these sites, it can be deduced that an origin of DNA replication is located in a region at, or close to, *ors12.* The results obtained with primer set A-A′ also suggest the existence of a second origin located approximately 1.7 kb upstream of *ors12* (A-A′ site).

When the abundance of nascent DNA strands at the same locus, and for equivalent fractions, was quantitated for CV-1 cells, a strong preference for initiation of replication at the *ors12* site (D-D′) was also observed (Fig. 6B), while the pattern obtained at the A-A′ site is again consistent with an origin being located upstream of *ors12.* Thus, with the shortest nascent DNA (average approx. 500 nt), the abundance of nascent DNA molecules was the greatest at the *ors12* site (Fig. 6B, D-D′, black bars) and was enriched by more than 10-fold by comparison to the segments B-B′, J-J′, M-M′, C-C′, and E-E′. Another peak was observed at the A-A′ segment, with approximately 56% of the abundance of molecules found at *ors12* (Fig. 6B, A-A′, black bars). When nascent DNA of longer size (approx. 1,200 nt) from the CV-1 nascent DNA preparation was tested, two major peaks were also observed, one at the *ors12* site (D-D′) and one at the A-A′ site (Fig. 6B, stippled bars), the latter having the highest abundance of nascent DNA molecules. Consistent with the increased amount of molecules at A-A′, the peak at the K-K′ segment, which is located close to the A-A′, was also increased. These data again suggest that an origin of bidirectional replication colocalizes with *ors12* in CV-1 chromosomes, while another one is located approximately 1.7 kb upstream of *ors12* (A-A′ site).

To further verify that the fractions of nascent DNA obtained from the sucrose gradient were not contaminated by randomly sheared parental DNA, a set of primers of unrelated sequence was generated outside the 8-kb genomic clone

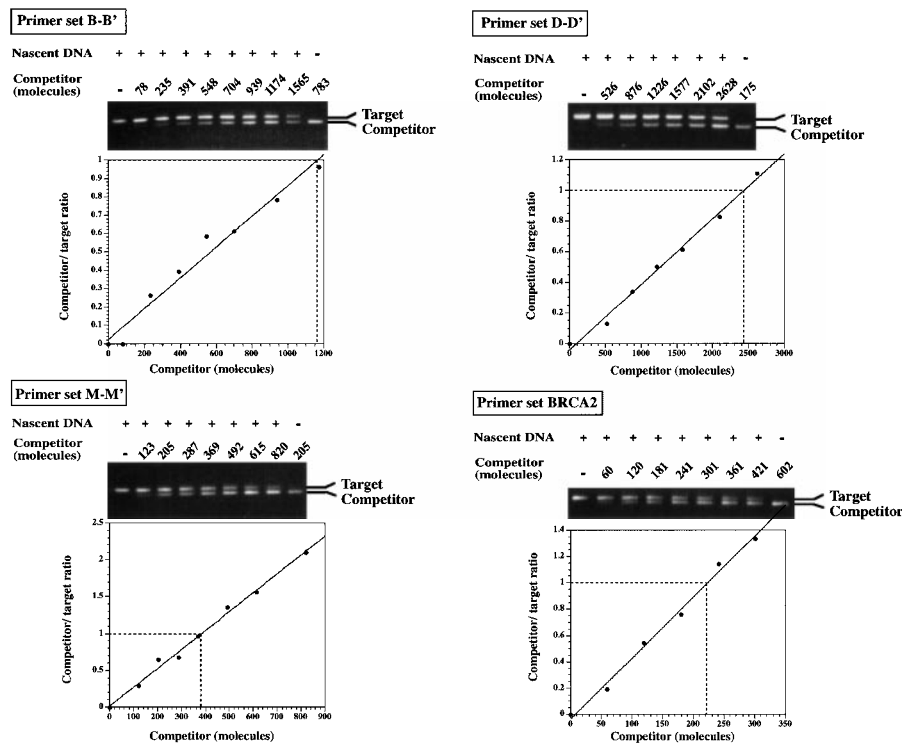

Fig. 5. Competitive PCR mapping analysis of *ors12* in AGMK cells. Nascent DNA was size-selected on a denaturing sucrose gradient and a fixed amount of each fraction was amplified with increasing amounts of a specific competitor for every primer set; the products were resolved on a 2% agarose gel, ethidium stained, and quantified. The bands corresponding to the nascent DNA (target) product and the competitor product, respectively, are indicated in each panel. The results were obtained with fraction #6 (lane 7, Fig. 4) from the sucrose gradient, containing newly synthesized molecules ranging in size between 250 nt and 950 nt (approximate average size of 600 nt)). The number of competitor molecules at a competitor/target ratio of 1 is indicated by a stippled line. The same procedure was followed for the other sets of primers that are not shown (K-K′, A-A′, J-J′, C-C′, and E-E′).

containing *ors12,* in the coding region of the African green monkey BRCA2 gene (Materials and Methods and Table I). These primers were used to amplify the nascent DNA from all fractions tested by competitive PCR. The results (Fig. 6, BRCA2) show that very few DNA strands from the BRCA2 genomic region are present in the nascent DNA preparation from AGMK and CV-1 cells. Thus, the enrichment found at the *ors12* locus and at the A-A′ site, in both CV-1 and AGMK nascent DNA, originates from specific initiation of DNA replication at that locus, and not from randomly degraded genomic DNA.

DISCUSSION

We previously generated from early replicating nascent DNA, isolated by extrusion [Zannis-Hadjopoulos et al., 1981] from replication bubbles of CV-1 cells [Kaufmann et al., 1985; Zannis-Hadjopoulos et al., 1985], a library of origin enriched sequences (*ors*). Several *ors* clones have been demonstrated to function as episomal origins of DNA replication in vivo [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Pelletier et al., 1997], and in a mammalian cell-free replication system [Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994; Pelletier et al., 1997]. Among the functional *ors, ors12,* a 812-bp-long sequence that contains 168-bp of the highly reiterated α-satellite sequence [Rao et al., 1990], has been mapped to the centromere of seven CV-1 cell chromosomes [Mah et al., 1992], and associates with the nuclear matrix in a cell-cycle dependent manner [Mah et al., 1993].

In this study, we used a low-copy ($<$9 copies per haploid genome of CV-1 cells) subfragment of *ors12* as probe to obtain a 14.2 kb CV-1 genomic fragment comprising *ors12,* which was then used for the in vivo mapping of *ors12* as a chromosomal origin of DNA replication. Two PCR-based origin-mapping methods were employed. First, a semi-quantitative PCR mapping method was used, whereby nascent DNA was amplified by PCR using primers located at

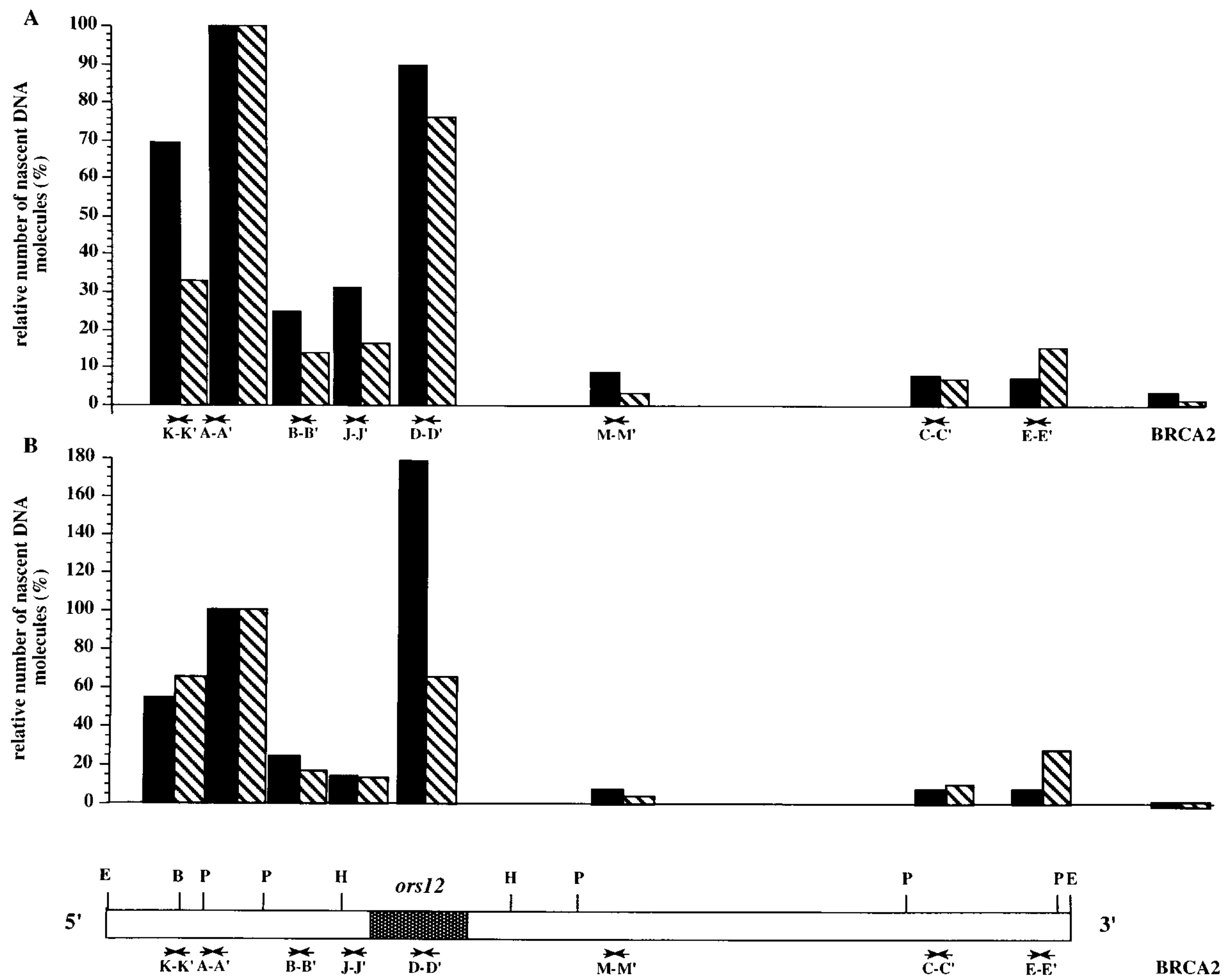

Fig. 6. Combined results of the competitive mapping experiments with different cell types. **A:** Histogram representing the relative abundance of nascent DNA molecules found at each PCR amplified segment in the genomic region under study. The quantification was done as explained in Figure 5 for the selected pools from the sucrose gradient containing AGMK nascent DNA: fraction #6 (250–950 nt, approximate average size of 600 nt; black bars) and fraction #7 (770–1,600 nt, approximate average size of 1,200 nt; stippled bars). The results are expressed relative to the amount of nascent DNA strands found at the A-A′ site, considered as 100%. As a control, the number of molecules found at the BRCA2 gene locus in the same pool is shown at the right of the map. **B:** Relative abundance of nascent DNA molecules found at the same locus in CV-1 cells, using pool #6 (175–770 nt, approximate average size of 500 nt; black bars) and pool #7 (750–1,600 nt, approximate average size of 1,200 nt; stippled bars) from the sucrose gradient. The results are expressed relative to the amount of nascent DNA strands found at *ors12* (D-D′ site; fraction #6) or the A-A′ segment (fraction #7), considered as 100%. The genomic map of the region under study is shown at the bottom.

different sites spanning the selected region containing *ors12* [Vassilev and Johnson, 1989]. The site of initiation was determined by the set of primers that amplified the shortest nascent DNA chains. We found that the shortest nascent DNA molecules were amplified by the set of primers located inside *ors12,* as well as by a set of primers located approximately 1.7 kb upstream of the *ors12* locus. This suggested that initiation of DNA replication in vivo occurs at a region comprising *ors12,* as well as at a second distinct site close to it. Because of the more limited quantitative nature of this method, a second, more quantitative, method was also employed. This method relies on the competitive PCR amplification of appropriately selected fragments spanning the putative origin region, and was recently used to precisely map the lamin B2 origin of DNA replication in human cells [Giacca et al., 1994; Kumar et al., 1996], the origin of DNA replication in the hamster DHFR gene domain [Pelizon et al., 1996; Kobayashi et al., 1998] and the human *c-myc* origin of replication [Tao et al., 1997].

Quantitation of the nascent molecules at each amplified segment can provide information on the frequency of initiation at each of those marker sites.

By this method, we found that the great majority of nascent DNA chains in AGMK and CV-1 DNA initiate replication at a site amplified by the primer set D-D′, which colocalizes with *ors12,* while the remaining molecules are distributed among DNA segments on either side of *ors12* (Fig. 6A,B). Consistent with the results obtained with the semi-quantitative PCR mapping procedure (Fig. 2B), another site of initiation was detected at the segment amplified by primers A-A′ (Fig. 6A,B), located approximately 1.7 kb upstream of *ors12*.

Detection of two or more sites of initiation in close proximity to one another has been observed before. Yoon et al. [1995] found that many sites of initiation are present in the intergenic region of the ribosomal RNA gene cluster in human cells, using a method that measures the abundance of nascent DNA strands at specific markers along the zone of initiation. Using 2D-gel mapping techniques, the same group found multiple initiation sites in the same locus in *S. cerevisiae* [Linskens and Huberman, 1988], while similar conclusions were reached for the same locus in human cells [Little et al., 1993]. Dijkwel and Hamlin [1995] also found that initiation occurs at a number of sites in the intergenic region in the amplified as well as in the nonamplified DHFR locus [Vaughn et al., 1990]. More recently, Kobayashi et al. [1998], using competitive PCR, reported three primary initiation sites present in the DHFR gene initiation zone. Two of these sites, oriβ and oriβ′, are located in close proximity, separated by approximately 5 kb of DNA. Using a PCR-based method, Shinomiya and Ina [1993] observed several initiation and pause sites in the 5 kb histone gene repeating unit of *Drosophila,* while 2D-gel and PCR methods revealed that DNA replication initiates at multiple sites in a zone of 10 kb downstream of the α-polymerase gene of *Drosophila* [Shinomiya and Ina, 1994]. Multiple initiation sites were also observed within a 6 to 8 kb zone in the chorion genes of *Drosophila* [Delidakis, 1989] and in the DNA puff II/9A of *Sciara coprophila* [Liang et al., 1993]. Finally, a recent report demonstrated that DNA replication initiates at several preferred sites in the vicinity of the *c-myc* gene [Waltz et al., 1996], which colocalize with an asymmetric pyrimidine heptanucleotide consensus sequence.

At least two explanations can be proposed for the results reported here. First, each of the two detected origins (*ors12* and the A-A′ segment) could be active at the same time and at the same locus, but in different cells; and second, the α-satellite sequence present at the 5′-end of *ors12* [Rao et al., 1990] may act as a replication fork barrier, thus blocking the 3′-elongation of chains initiated at the A-A′ segment and the 5′-elongation of chains initiated at *ors12.* It has been previously suggested that α-satellite containing sequences may be activated early in S phase, but their replication may then be arrested until later in S-phase [Matsumoto and Gerbi, 1982; Landry and Zannis-Hadjopoulos, 1991; Mah et al., 1992]. In support of this hypothesis, it was reported that in the yeast *S. cerevisiae,* replication forks pause at all centomeres analysed [Greenfeder and Newlon, 1992].

The competitive PCR method permits the study of initiation of DNA replication in complex genomes without prior need for cell synchronization using inhibitors, thus avoiding the introduction of any bias that might arise from the cell synchronization procedure, and making it possible to look at origin activation under physiological conditions. Furthermore, by omitting the labeling of nascent DNA with BrdU and subsequent precipitation with anti-BrdU antibodies [Vassilev and Johnson, 1989], the likelihood of DNA photo-damage and loss of sample, which would introduce a bias in the subsequent quantitation by competitive PCR, was reduced to a minimum. The validity of this approach was recently demonstrated in two independant studies in which control primers located in the human β-globin gene were used to amplify nascent DNA prepared from different human cell lines, for the mapping of the lamin B2 [Giacca et al., 1994; Kumar et al., 1996] and the *c-myc* [Tao et al., 1997] origins of replication. Consistent with the results presented here, the abundance of molecules containing a segment of the β-globin gene in the nascent DNA sample was similar to the markers with the lowest abundance in the lamin B2 region [Giacca et al., 1994; Kumar et al., 1996]; similarly, amplification of the *c-myc* origin was not due to random degradation of the genomic DNA [Tao et al., 1997].

In summary, the data demonstrate that replication initiates nonrandomly in the 8-kb region

that comprises the *ors12* locus in CV-1 and AGMK nascent DNA. This is the first demonstration of chromosomal origin function for an autonomously replicating *ors* sequence isolated by the nascent strand extrusion method [Zannis-Hadjopoulos et al., 1981]. Taken together with previously published data [Frappier and Zannis-Hadjopoulos, 1987; Pearson et al., 1991; Zannis-Hadjopoulos et al., 1994], the present results also show that *ors12* can act both as a chromosomal origin and as an autonomously replicating element.

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