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Distribution of DNA Replication Origins Between Matrix-Attached and Loop DNA in Mammalian Cells

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Abstract Using a previously developed procedure (Gencheva et al. [1996] J Biol Chem 271:2608–2614), we isolated a DNA fraction consisting of short fragments originating from the regions of initiation of DNA synthesis from exponentially growing Chinese hamster ovary cells. This fraction arbitrarily designated as ''collective origin fraction'' was labeled in vitro and used to probe the abundance of origin containing sequences in preparations of matrix-attached and loop DNA isolated by two different procedures from Chinese hamster ovary cells. Alternatively, an individual DNA replication origin sequence — a 478-bp long DNA fragment located at about 17-kb downstream of the dihydrofolate reductase gene — was used to probe the same matrix-attached and loop DNA fractions. The results with both the collective and individual DNA replication origins showed that there was random distribution of the origin sequences between DNA attached to the matrix and DNA from the loops. J. Cell. Biochem. 80:353–359, 2001.
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Key words: replication origin; nuclear matrix; loop DNA; matrix-attached DNA; DHFR ori-β; replication bubble trap

Eukaryotic DNA is organized into about 10^4 tandemly arranged functional DNA domains called replicons. Each replicon replicates from a single replication origin in a regulated way during the S-phase, only once per cell cycle. Early data have shown that groups of replication origins are coordinately controlled and fire in clusters [Huberman and Riggs, 1968]. Recent studies demonstrated that these clusters represent morphological structures called replication foci, sites or centers that persist throughout the cell cycle and in the subsequent daughter cells. These clusters serve to coordinate and regulate the activation of multiple adjacent origin sites [Newport and Yan, 1996; Jackson and Pombo, 1998; Ma et al., 1998; Dimitrova and Gilbert, 1999]. Biochemical studies have identified many proteins that are essential for DNA replication in eukaryotic cells. However, thus far initiation of DNA synthesis at specific sequences in mammalian cells can only be observed in intact nuclei. In the in vitro replication system using *Xenopus* egg extract DNA replication is initiated at specific sites, when the DNA substrate is in the form of intact nuclei and initiation is random, when DNA is presented in protein-free form [Gilbert et al., 1993, 1995]. These experiments suggest that initiation sites for DNA replication in mammalian cells are composed of specific DNA sequences organized in a nucleoprotein structure that includes components of nuclear structure [DePamphilis, 1999]. A possible candidate for a nuclear structure that may take part in the establishment of initiation sites for DNA replication is the nuclear matrix. The nuclear matrix, scaffold, or skeleton is mainly a proteinaceous structure isolated by treating nuclei with nonionic detergents, nucleases, and solutions of high ionic strength [Berezney et al., 1995]. It has been demonstrated that there is a remarkable preservation of replication foci and their S-phase specific pattern after extraction for nuclear matrix [Nakayasu and Berezney, 1989]. The organization of chromatin into replication foci is considered to be mediated by the clustering of repeating 50–200 kb loops attached to the nuclear matrix [Berezney et al., 1995; Kunnev et al., 1997]. Pulse-chase labeling experiments

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have shown that DNA attached to the matrix is the first to be labeled. Shortly after the pulse, the newly synthesized DNA is chased away from the matrix [Berezney and Coffey, 1975; Berezney and Buchholtz, 1981; Pardoll et al., 1980; Vogelstein et al., 1980; Dijkwel et al., 1979; Jackson and Cook, 1986; Vaughn et al., 1990; Ortega and DePamphilis, 1998]. These results indicated that the newly synthesized DNA is attached to the nuclear matrix and now it is unanimously accepted that replication forks are associated with the nuclear matrix. The question whether replication origins are associated with the nuclear matrix is still open. A model has been proposed according to which replication forks do not travel along DNA, but rather DNA is being reeled through huge and complex fixed structures called replication factories, where it is replicated [Jackson, 1990; Hozak et al., 1993]. That model does not require permanent attachment of replication origins to the nuclear matrix and predicts only a transient attachment. On the other hand, there are results showing that replication origins may be permanently attached to the nuclear matrix [Aelen et al., 1983; Van der Velden et al., 1984a, 1984b; Dijkwel et al., 1986; Razin et al., 1986; Carri et al., 1986; Kalandadze et al., 1990; Lagarkova et al., 1998]. Accordingly, models have been proposed to explain how a DNA loop anchored at a nuclear structure could replicate by reeling through the anchoring site and the region where replication had begun to remain attached to the same site [Dingman, 1974; Cook, 1991; Newport and Yan, 1996].

In the present paper, we have fractionated DNA of Chinese hamster ovary (CHO) cells into two fractions, one enriched in 'loop' DNA and the other enriched in 'matrix-attached' DNA. We have analyzed the distribution of different origin sequences between these two fractions and have found that there is no preferential association of the origin sequences to the matrix. The results speak against the possibility that DNA initiation regions are permanently attached to the nuclear matrix during the entire cell cycle.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary cells (CHO K1) were grown in Eagle's MEM supplemented with 10% fetal bovine serum (Sigma) in an atmosphere of 96% air/4% CO_2.

Isolation of the Collective Origin Fraction

The collective origin fraction was isolated as described by Gencheva et al. [1996]. DNA was crosslinked in vivo by four successive treatments with Trioxsalen and near UV light to give one Trioxsalen bridge per 1.5 kb on the average. The cells were incubated in the presence of 50 μM BrdUrd (Sigma) and 20 μCi/ml [^{3}H] dC (20–40 Ci/mmol, DuPont) for 1 h to label the nascent DNA fragments synthesized between crosslinks. Cells were lysed in 0.5% SDS, 1 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH 8 and the proteins were digested with 200 μg/ml Proteinase K (Merck) at 37°C for 4 h. After treatment with phenol-chloroform (1:1) and chloroform, 1 volume of ethanol was overlayered and the high molecular weight chromosomal DNA was recovered by spooling on a glass rod. DNA was dissolved in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4 to give 200–500 μg DNA/ml, made 0.2 M in NaOH by adding 1 M NaOH, and centrifuged in 5–20% sucrose density gradients prepared in 0.2 M NaOH, 1 mM EDTA in Beckman SW 27 rotor at 25,000 rpm, 10°C, for 18 h. Aliquots were counted, the fractions containing the nascent DNA chains were pooled together and precipitated with ethanol. DNA was dissolved in 0.14 M NaCl, 0.01 M phosphate buffer, pH 7 containing 0.5% Tween 20 and 100 μg/ml bovine serum albumin in a final volume of 400 μl. An equal volume of monoclonal anti-bromodeoxyuridine antibody (Beckton and Dickinson) was added, and after 1 h at room temperature, the antigen–antibody complex was precipitated with an excess of second antibody (anti-mouse IgG rabbit IgG fraction, Sigma). After another hour at room temperature, the samples were kept at 4°C overnight and the precipitate was collected by centrifugation in an Eppendorf microcentrifuge for 10 min. It was washed with 0.14 M NaCl, 0.01 M phosphate buffer, pH 7 and deproteinized.

Isolation of the 478 kb DNA Fragment from the DHFR Replication Origin

CHO genomic DNA was used as template to generate a 478-bp long DNA fragment (from nucleotide 888 to nucleotide 1366 in the sequence, Leu et al., 1990) by PCR. Primers were 5′-TCGGCCTGTCTGTAATATTT-3′ and

5′-CTGTGGAGCTGCTGTGTTTT-3′. The parameters of the PCR cycle were denaturation at 96°C for 30 s, annealing at 72°C for 30 s, and synthesis at 66°C for 1 min. The generated fragment was purified by electrophoresis in 1% agarose gel and cloned in pBluescript II (KS+) according to standard procedures [Ausubel et al., 1992].

Isolation of Matrix-Attached and Loops DNA by Digestion with Restriction Endonucleases

DNA from the bases of the loops and the loops was isolated essentially as described by Robinson et al. [1983]. Cells were suspended to give about 10^8 cells/ml in LS buffer (100 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM PMSF, 20 mM Tris, pH 7.2) containing 1% Nonidet P40. After centrifugation at 600*g* for 5 min, the pellet was resuspended in the same buffer and was homogenized by 10 strokes in a Dounce homogenizer, Type A. Nuclei were obtained by centrifugation at 600*g* for 5 min and were resuspended in LS buffer to a concentration of 10^7 nuclei/ml. The suspension was adjusted to 2 M NaCl by addition of equal volume of 4 M NaCl, 5 mM $MgCl_2$, 0.5 mM PMSF, 20 mM Tris, pH 7.2 and was layered on top of a cushion of 50% glycerol in HS buffer (2 M NaCl, 5 mM $MgCl_2$, 20 mM Tris, pH 7.2). After centrifugation at 4,000*g* for 40 min, the nuclear halos were collected from the interphase and were pelleted at 20,000*g* for 20 min. The pellet was suspended in 20 vol. of 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 100 mM Tris, pH 7.2 and was incubated with 1,200 U *Bam*HI and 1,200 U *Hind*III for 3 h at 37°C. After the digestion, the matrix-bound DNA was pelleted down by centrifugation at 10,000*g* for 20 min, while the DNA loops remained in the supernatant. Both DNA fractions were incubated with 200 μg/ml Proteinase K at 37°C for 4 h in buffer containing 0.5 M NaCl, 20 mM EDTA, 0.5% SDS, 25 mM Tris–HCl, pH 8. RNA was removed by treatment with 20 μg/ml of RNase A. After deproteinization with phenol-chloroform (1:1) and chloroform, DNA was precipitated with 2.5 volume of ethanol.

Isolation of Matrix-Attached DNA by DNAse I Treatment

The matrix-attached DNA was isolated essentially as described by Cockerill and Garrard [1986]. Cells were suspended in RSB-sucrose (0.24 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF, 10 mM Tris, pH 7.4) and 0.5% Triton X100 to make 10^7 cells/ml and homogenized with 10 strokes in a Dounce homogenizer. After 10 min on ice nuclei were pelleted by centrifugation at 1,000*g* for 10 min, washed in 5 ml of RSB-sucrose, and dissolved in the same buffer. The nuclei were purified by centrifugation through an equal volume of RSB containing 0.5 M sucrose at 1,400*g* for 15 min. The pellet was dissolved in RSB-sucrose to give about 10^8 nuclei/ml and was incubated with 15 μg/ml DNAse I (Boehringer) for 20 min at room temperature. The suspension was centrifuged at 3500 rpm in an Ependorf centrifuge for 10 min and the pellet was dissolved in 2 M NaCl, 20 mM EDTA, 20 mM Tris, pH 7.4. After 10 min on ice, the matrix fraction was collected by centrifugation at 3,500 rpm for 10 min and the matrix-attached DNA was deproteinized as described above.

Labeling and Hybridization

DNA probes were labeled with [^{32}P]dCTP (DuPont, 3,000 Ci/mmol) using RadPrime DNA Labeling System (GibcoBRL). For dot-blot hybridization, DNA was loaded onto Hybond-N+ membrane (Amersham) as recommended by the manufacturer using a manifold dot-blotter (BioRad). Hybridization was carried out under stringent conditions (7% SDS, 0.25 M phosphate buffer, 1% bovine serum albumin, at 68°C overnight). When the collective origin fraction was used as a probe, 10 μg/ml of non-labeled sonicated genomic CHO DNA was added to the prehybridization and hybridization solutions. The membranes were rinsed with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7) at room temperature (twice), washed with 2 × SSC, 0.1% SDS at 68°C for 30 min, then in 0.2 × SSC, 0.1% SDS at 68°C for 30 min and finally rinsed with 0.1 × SSC at room temperature.

RESULTS AND DISCUSSION

A possible way to determine whether origins of mammalian DNA replication are permanently associated with the nuclear matrix is to fractionate nuclear DNA into DNA of the loops and DNA of the bases of the loops that is attached to the matrix, and to determine the abundance of origin sequences in these fractions. Matrix-attached DNA was obtained by

two methods, differing in the order of treatment of the nuclei with high salt and nucleases. After removing histones and most of the nonhistone proteins with 2 M NaCl, DNA loops out to form structures called halos. These structures were purified and digested with the restriction endonucleases *Hind*III and *Bam*HI. About 80% of the total DNA were solubilized by this treatment, while the remaining 20% remained attached to the residual nuclear structures. They were pelleted down and the attached DNA, representing DNA of the bases of the loops, was isolated from the pellet, while DNA of the loops was isolated from the supernatant. In another series of experiments, nuclear matrices were isolated by treatment of nuclei first with DNAse I and then with 2 M NaCl. The obtained residual structures contained about 10% of the genomic DNA representing matrix-attached DNA. In this case, the loop DNA was not possible to isolate since it has been digested by the DNAse I treatment.

We isolated two types of DNA replication origin preparations. The first one represented a mixture of nascent DNA fragments containing different replication origins. Briefly, exponentially proliferating cells were treated with trioxsalen to crosslink DNA in vivo. Then the cells were cultured with BrdU for 1 h. During this period, DNA synthesis begins at some origins situated between the crosslinks, but the growing nascent chains cannot pass through the trioxsalen bridges and their movement is effectively blocked after traveling a few hundred base pairs. This leads to the accumulation of short nascent DNA fragments containing sites of initiation of DNA synthesis, which are not ligated to DNA and can be easily isolated as a light peak by alkaline sucrose density gradient centrifugation. The fraction enriched in replication origins was further purified by immunoprecipitation with anti-bromodeoxyuridine antibody. The procedure is known in the literature as the "replication bubble trap" for isolation of replication origins [Kobayashi et al., 1998]. It has been used to map the origins of replication in the DHFR domain of CHO cells [Anachkova and Hamlin, 1989] and in the rDNA repeats in the human and mouse genomes [Gencheva et al., 1996], and to clone and characterize mouse replication origins [Dimitrova et al., 1993]. The isolated DNA preparation designated here as "the collective origin fraction" (Fig. 1) was further deproteinized, labeled in vitro and used as a probe.

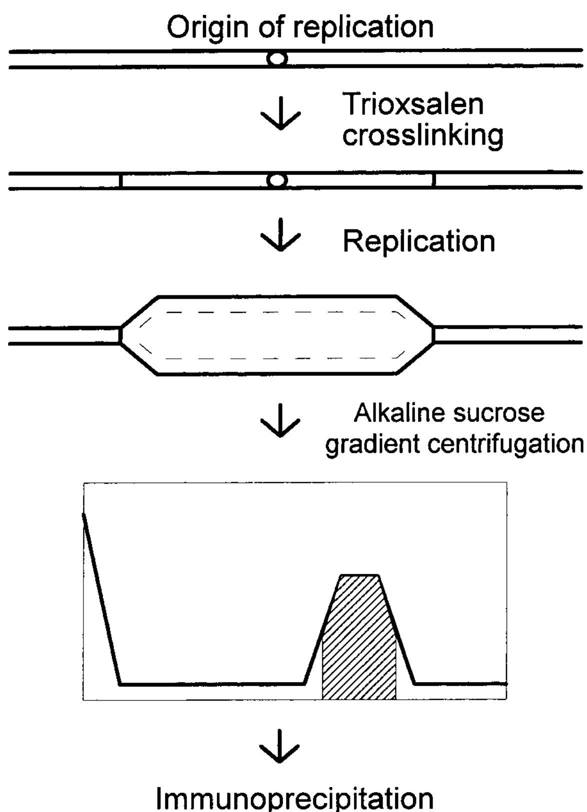

Fig. 1. Schematic representation of the procedure for isolation of the collective origin fraction.

The second probe represented a unique sequence originating from the region of the origin of replication located downstream of the 3′-end of the DHFR gene in CHO cells, designated as ori-β (Fig. 2). Three preferred initiation sites have been identified in the initiation zone encompassing the nontranscribed spacer

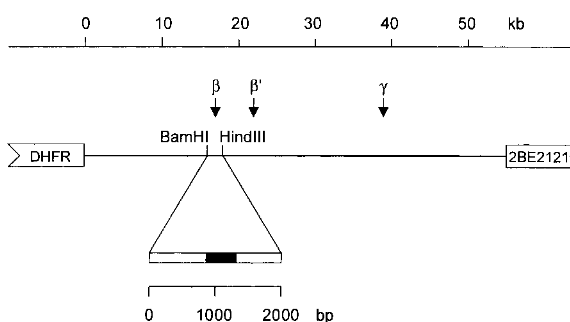

Fig. 2. Schematic representation of the nontranscribed spacer 3′ of the DHFR gene in CHO cells. The spacer contains three origins of DNA replication designated as ori-β, ori-β′ and ori-γ. A 478 bp DNA fragment (filled box) was amplified by PCR and used as a probe.

3′- of the DHFR gene — ori-β, ori-β′ and ori-γ — of which ori-β is the primary initiation site and one of the most thoroughly studied mammalian origins so far [Kobayashi et al., 1998]. Several different methods for mapping replication origins reveal that initiation occurs in an 1.96 kb *Bam*HI/*Hind*III fragment located at about 17-kb downstream of the DHFR gene: the replication bubble trap [Anachkova and Hamlin, 1989]; Okazaki fragments distribution [Burhans et al., 1990]; leading strength distribution [Handeli et al., 1989; Burhans et al., 1991]; earliest labeled DNA fragments [Heintz and Hamlin, 1982; Burhans et al., 1986; Leu and Hamlin, 1989; Li et al., 2000]; nascent DNA strands length [Vassilev et al., 1990]; and nascent DNA strands abundance [Pelizon et al., 1996; Kobayashi et al., 1998]. A 478-bp long unique DNA sequence within ori-β was generated by PCR, cloned and used as a probe.

To analyze the distribution of origin sequences between the matrix-attached DNA and loop DNA, they were dot blotted and hybridized with either the in vitro labeled 'collective origin DNA' (Fig. 3A), or with the 478-kb DNA fragment, derived from the region of the DHFR ori-β (Fig. 3B). The abundance of replication origin sequences in the matrix-attached and loop DNA was compared with the abundance of those sequences in genomic DNA digested with *Hind*III and *Bam*HI. If origins were permanently bound to the nuclear matrix, a 5–10-fold enrichment of the origin sequences should be expected in the matrix-attached DNA. The results showed that there was no difference in the intensity of the hybridization signals between the matrix-attached DNA, the loop DNA and genomic DNA with either of the origin probes. This means that there were no differences in the distribution of the origin sequences in the three DNA preparations and can be interpreted as lack of preferential association of replication origins with the nuclear matrix. However, as we have worked with exponentially growing cells it cannot be ruled out that the origins may be preferentially bound to the nuclear matrix only during a short period of the cell cycle. Thus, our results do not contradict the hypothesis of a transient association of replication origins with the nuclear matrix. Recently, two papers appeared that present opposing results regarding the problem. Using a procedure for topoisomerase II-mediated DNA loop excision, Lagarkova et al. [1998] have shown that in exponentially growing mammalian cells replication origins are located at DNA loop anchorage regions and corroborated the supposition for permanent attachment of the origins to the nuclear matrix. Using nucleoskeletons prepared by encapsulating cells in agarose and extracting them with a nonionic detergent in a physiological buffer, Ortega and DePamphilis [1998] have not found any preferential association of the DHFR origins of replication to the nuclear skeleton during G1- and S-phase of the cell cycle. Our data based on experiments using the classical methods for isolation of the nuclear matrix with high salt solutions confirm the results of Ortega and DePamphilis [1998]. Taken together these results do not support the possibility that DNA initiation regions may be permanently attached to the nuclear matrix during the entire cell cycle.

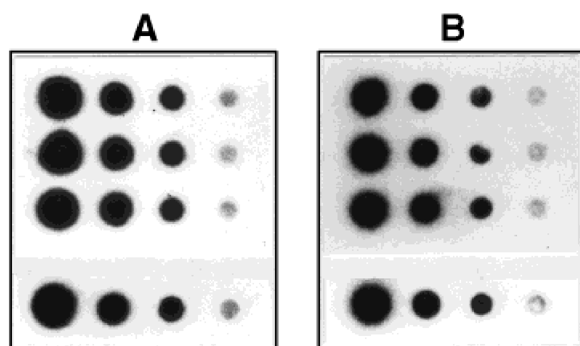

Fig. 3. Matrix-attached DNA prepared by DNase I digestion (first row), matrix-attached DNA prepared by restriction endonuclease digestion (second row), loop DNA prepared by restriction endonuclease digestion (third row), and genomic DNA (fourth row) were dot blotted on membranes in decreasing concentrations (10 μg, 1 μg, 0.1 μg, and 0.01 μg per dot). The membranes were hybridized with the labeled in vitro with ^{32}P collective origin fraction (**A**) and with the 478 bp DHFR ori-β probe (**B**).

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