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DNase I Sensitive Site in the Core Region of the Human β-globin Origin of Replication

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Abstract HeLa cells were synchronized at late G1, early S, and late S phase of the cell cycle by nocodazole treatment. The cells were permeabilized with Triton X-100, digested with DNAse I, and extracted with 0.2 M ammonium sulfate to remove the digested chromatin. DNA was isolated from the residual chromatin attached to the nuclear matrix, digested with Hind III, and subjected to hybridization with [^{32}P] labeled probe located upstream of the core region of the human β-globin replication origin. The hybridization pattern revealed the existence of a DNase I sensitive site in the core region of the β-globin replicator. The results suggest that association with the nuclear matrix induce alteration in the chromatin structure of the origin of replication that represents a more open chromatin configuration. J. Cell. Biochem. 87: 279–283, 2002. © 2002 Wiley-Liss, Inc.

Key words: human β-globin origin of replication; nuclear matrix; replication foci; DNase I sensitive site; indirect end labeling; cell cycle

A characteristic feature of mammalian DNA replication is that it occurs at a few hundred discrete foci. A typical replication focus contains a cluster of several replicons that fire simultaneously. Replicon clusters are stable units of chromosomal structure and persist throughout the cell cycle and in the subsequent daughter cells [Jackson and Pombo, 1998; Ma et al., 1998; Dimitrova and Gilbert, 1999]. The replication foci exhibit specific patterns during S phase and these patterns are remarkably preserved after extractions for nuclear matrix [Nakayassu and Berezney, 1989; Ma et al., 1999]. That is why it is considered that replication foci represent a higher order chromatin structure formed by the aggregation of several 50–200 kb DNA loops attached to the nuclear matrix [Kunnev et al., 1997; Berezney et al., 2000]. The nuclear matrix is mainly a proteinaceous structure isolated by treating nuclei with nonionic detergents, nucleases, and solutions of moderate and high ionic strength [Berezney et al., 1995]. Recently, we studied the cell cycle dependence of the association and dissociation of origins of replication with replication foci attached to the nuclear matrix. The results showed that replication origins are attached to the matrix in late G1 phase and detached after initiation of DNA replication in S phase [Djeliova et al., 2001]. Pemov et al. [1998] have shown that MNase-hypersensitive sites appeared in the Chinese hamster origins of replication located downstream of the dihydrofolate reductase (DHFR) gene at the G1/S boundary, but only in those copies of the DHFR amplicon that are attached to the nuclear matrix in CHOC 400 cells. These MNase hypersensitive sites disappeared in early S phase and the authors suggested that a temporal attachment of the origin regions to the matrix at the G1/S boundary of the cell cycle modulate chromatin architecture to facilitate the activation of the origins in S phase. To see whether alteration of the chromatin structure of origins of replication upon attachment to the nuclear matrix is a general characteristic of mammalian origins, in the present communication we studied the DNase I sensitivity in the human β-globin origin of replication. To this end, replication foci attached to the nuclear

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matrix were isolated from HeLa cells synchronized around the G1/S boundary and S phase. DNA attached to the nuclear matrix was isolated, digested with a restriction nuclease, and the presence of DNase I-sensitive sites determined by indirect end labeling. The results revealed an unregistered by now DNase I-sensitive site in the core region of the human β-globin origin of replication.

MATERIALS AND METHODS

Cell Cultures, Labeling, and Synchronization

Human HeLa (HeLa M) cells were grown in DMEM supplemented with 10% fetal bovine serum in an atmosphere of 96% air/4% CO_2. Cells were synchronized in mitosis by incubating 70% confluent cultures for 4 h with 50 ng/ml nocodazole. Mitotic cells were collected, washed free of nocodazole, and cultured under normal conditions. For fluorescence activated cell sorting (FACS) analysis cells were collected, washed with phosphate buffered saline, treated with 20 μg/ml RNase for 30 min at 37°C, and stained with 20 μg/ml propidium iodide at room temperature for 30 min. A total of 5×10^5 cells/sample were analyzed with a Becton Dickinson (Facscalibur) cell sorter, using ModFit software (Becton Dickinson).

Preparation of Replication Foci Attached to the Nuclear Matrix

Cells were washed twice with TBS buffer (0.15 M NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). They were permeabilized in situ with 0.1% Triton X-100 in the presence of 0.5 mM phenymethylsulfonyl fluoride (PMSF) in TBS at room temperature for 5 min, followed by a wash with TBS buffer. Replication foci attached to the nuclear matrix were prepared essentially as described by Nakayassu and Berezney [1989]. The chromatin of the permeabilized cells was digested in situ with 7 U/ml of DNase I (Sigma-Aldrich) in TBS buffer at room temperature for 7 min and extracted twice with 20 mM Tris-HCl, pH 7.4, containing 0.2 M ammonium sulfate, and 0.2 mM $MgCl_2$ at room temperature for 1 min. The samples were then washed twice with TBS buffer. Under this conditions about 30–40% of total DNA remained in extracted nuclei.

Isolation and Electrophoresis of DNA

For DNA isolation, cells were lysed in 0.5 M NaCl, 0.5% sodium dodecylsulfate, 20 mM EDTA, 25 mM Tris-HCl, pH 8. RNA was degraded with 200 μg/ml RNase at 37°C for 3 h. Proteins were digested with 200 μg/ml Proteinase K (Sigma) overnight, and after extraction with phenol–chloroform (1:1) and with chloroform–isoamyl alcohol (24:1), DNA was precipitated with 2.5 volumes of ethanol. It was dissolved in 1 mM EDTA, 10 mM Tris-HCl, pH 8, and DNA concentration was determined by reading the optical density at 260 nm. Isolated naked DNA was digested with 0.00025 U DNase I/μg DNA in 50 mM Tris/10 mM Mg Cl_2, pH 7.5 buffer. DNA isolated from residual nuclei and naked DNA digested with Dnase I was completely digested with Hind III. DNA fragments were separated on 1% agarose gel in 40 mM Tris-acetic acid, 2 mM EDTA, pH 8, and the gels were stained with ethidium bromide. The molecular weight marker used was 1 kb DNA Ladder (Gibco BRL, Grand Island, NY).

Hybridization

For hybridization gels were placed in denaturation solution (1.5 M NaCl/0.5 M NaOH) for 30 min, washed with ddH_2O, after which washed twice for 15 min in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M EDTA) and then transferred to Hybond N+ membrane (Amersham). The blotting was performed with 10 × SSC (1.5 M NaCl/0.15 M sodium citrate, pH 7.0) for 16 h at room temperature. The Hybond N+ membrane was washed gently with denaturation solution for 1 min and twice with neutralization solution for 30 s. The membrane was dried for 1 h at 80°C. The human β-globin probe representing a 696 bp Bam HI/Bg1II fragment (position 59881–60577 in the hβG locus, GenBank accession number J00179) located upstream of the β-globin gene was kindly donated by H. Cedar [Probe H in Kitsberg et al., 1993]. The probe was labeled in vitro with [^{32}P] dCTP (DuPont, 3,000 Ci/mmol) using RadPrime DNA Labeling System (Gibco BRL). Hybridization was carried out under stringent conditions (7% SDS, 0.25 M phosphate buffer, 1% bovine serum albumin at 68°C overnight). 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 (twice), then in 0.1 × SSC, 0.1% SDS at room temperature for 30 min and finally rinsed with 0.1 × SSC at room temperature.

RESULTS AND DISCUSSION

The β-globin gene is not expressed in HeLa cells and is replicated in late S phase [Taljanidisz et al., 1989; Kitsberg et al., 1993]. We have previously shown that the late firing β-globin origin of replication is associated with the nuclear matrix in late G1 and stays attached until replication is initiated in late S phase [Djeliova et al., 2001]. To study the chromatin structure of the human β-globin origin of replication during the period when it is attached with the nuclear matrix, HeLa cells were synchronized at mitosis by nocodazole treatment. Cells were collected at the 8th, 11th, and 15th h after release from the nocodazole block, which corresponded to late G1 phase and early and late S phase [Djeliova et al., 2001]. The FACS analysis confirmed that at the 8th h over 80% of the cells were in G1 phase and at the 11th hour about 70% of the cells were in S phase.

To isolate pre-replication and replication foci attached to the nuclear matrix we used the method of Nakayassu and Berezney, 1989. The method includes permeabilization of the cells in situ with the detergent Triton X-100, followed by digestion of the loop DNA with DNase I and extraction under mild conditions with 0.2 M ammonium sulfate. The latter effectively extracts the digested chromatin, while having no detrimental effect on the residual chromatin structures [Ma et al., 1999]. The residual structures are capable of synthesizing DNA without the addition of nuclear and cytoplasmic extract [Nakayassu and Berezney, 1989; Djeliova et al., 2001]. The pattern of replication foci visualized by labeling in vitro is indistinguishable from the S phase specific pattern visualized in intact cells by in vivo labeling [Nakayassu and Berezney, 1989]. This indicates that the in situ prepared replication foci anchored at the nuclear matrix are preserved functionally active during the isolation procedure. That opens the possibility to analyze the structure of the chromatin attached to the nuclear matrix.

The chromatin attached to the nuclear matrix was studied by in vivo footprinting with DNase I. In this case DNase I simultaneously served to digest matrix-distal loop DNA and cleave matrix-proximal DNA. The digested loop DNA (60–70% of total DNA) was extracted with 0.2 ammonium sulfate. DNA attached to the nuclear matrix was purified and digested to completion with the restriction nuclease HindIII. The human β-globin origin of replication has been mapped within a 7.8 kb HindIII fragment by three biochemical methods—the replication direction of the leading strands [Kitsberg et al., 1993], the short nascent DNA strands abundance [Aladjem et al., 1998], and the lambda exonuclease-resistant nascent strands [Cimbora et al., 2000]. This origin is one of the most thoroughly studied mammalian origins of replication so far and solid genetic evidence has been presented that initiation occurs at a specific DNA sequence [Kitsberg et al., 1993; Aladjem et al., 1998]. By using a combination of site-specific recombination and physical mapping technique Aladjem et al. [1998] have shown that the β-globin origin consists of a 2.6 kb long core replicator region and two auxiliary elements (Fig. 1).

To locate the DNase I-sensitive sites within the core region we have used the method of indirect end labeling [Nedospasov and Georgiev, 1980; Wu, 1980]. DNA was electrophoresed and blotted on a nylon membrane. The fragment used as a hybridization probe was a 696 bp Bam HI/Bg1II fragment located upstream of the core replicator region and near the 5′-end of the HindIII fragment (Fig. 1). The results are presented in Figure 2A. It is seen that at late G1 phase, early and late S phase there is a strong preference for DNase I cleaving at a site located about 3 kb downstream of the 5′-end of the HindIII fragment. It should be noted that no such DNase I sensitive site was registered when

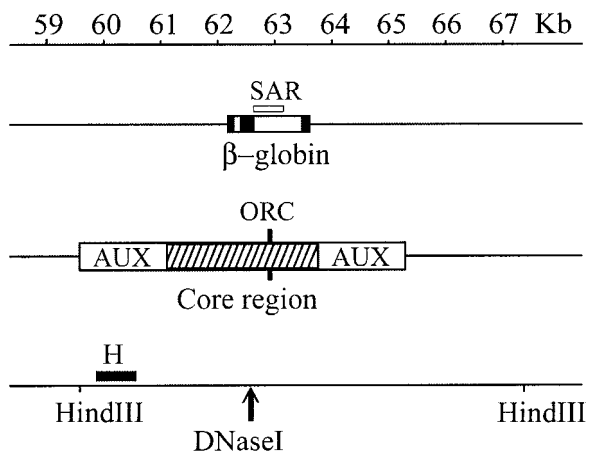

Fig. 1. Physical map of the β-globin gene and the β-globin origin of replication. Indicated are the positions of the SAR, the consensus binding site of ORC, the DNA probe H, and the Hind III restriction sites used to map the DNase I sensitive site (arrowhead).

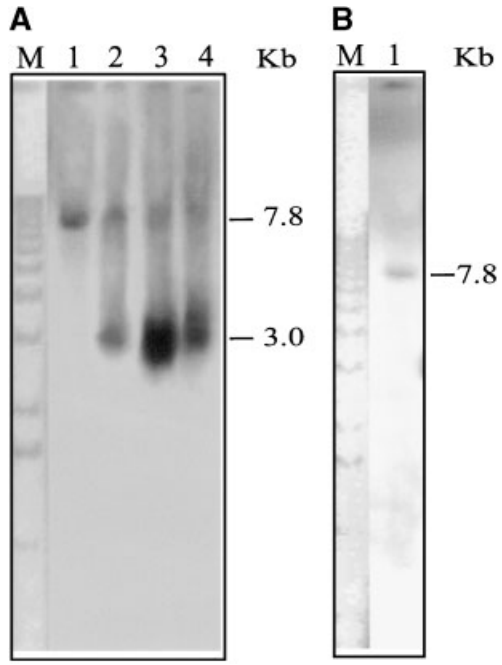

Fig. 2. Mapping the DNAse I sensitive site. **A**: HeLa cells were synchronized by nocodazole; 8, 11, and 15 h after release from the nocodazole block cells were permeabilized with Triton X-100, digested with DNase I, and extracted to obtain replication foci attached to the nuclear matrix. DNA was isolated, digested with HindIII, and electrophoresed. DNA fragments were transferred to nylon membranes and hybridized with the in vitro [^{32}P] labeled DNA probe H. M, molecular weight marker; **lane 1**: HeLa genomic DNA digested with HindIII; **lanes 2**, **3**, and **4** represent DNA attached to the matrix isolated 8, 11, and 15 h after release from the nocodazole block, respectively. **B**: Genomic HeLa DNA was digested with DNase I and HindIII, electrophoresed, transferred onto nylon membrane, and hybridized with the same radioactive probe.

the experiment was carried out on purified genomic DNA digested with DNase I to the same extent as in the permeabilized cells (Fig. 2B). This means that the cleavage was not dependent on the DNA sequence, but on the chromatin structure of the origin region.

The results reveal a DNase I sensitive site in the core region of the human β-globin origin of replication (Fig. 1) suggesting that one or two nucleosomes have been disrupted [Gross and Garrard, 1988]. The site is located at the 3′-end of the second exon of the β-globin gene and abuts a long A/T rich sequence region in the second intron of the gene. The A/T rich sequence contains 520 bp long scaffold attachment region (SAR) [Jarman and Higgs, 1988] within which a consensus binding site for the origin recognition complex (ORC) have been found [Bogan et al., 2000].

The chromatin structure of the β-globin gene in HeLa cells has been investigated before with DNase I and no hypersensitive sites have been detected. However, the experiments were carried out with exponentially growing cells and on whole nuclei [Groudine et al., 1983; Cao and Schechter, 1988; Dhar et al., 1988] and that is why the registered in the present communication DNase I sensitive site has probably not been revealed. Our results are consistent with the results of Pemov et al. [1998] who have found a MNase sensitive site in the DHFR ori-β at the G1/S boundary of the cell cycle in the copies of the DHFR amplicon that are attached to the nuclear matrix. The DHFR gene is a housekeeping gene and replicates in early S phase [Taljanidisz et al., 1989; Kitsberg et al., 1993]. The β-globin gene is not expressed in HeLa cells and the β-globin origin fires at late S phase. Our previous results have shown that while ori-β is attached to the matrix only for a short period at the border G1/S, the β-globin origin is attached to the matrix in late G1 and stays attached until late S phase [Djeliova et al., 2001]. Accordingly, the alteration in the chromatin structure registered as DNase I sensitive site persists through S phase until DNA replication is initiated. There are data that another well-characterized origin of replication, the one located downstream of the human lamin B2 gene, is mapped to a matrix anchorage site [Lagarkova et al., 1998]. By using high-resolution in vivo footprinting a prominent protein protection was detected at the lamin B2 origin of DNA replication. The footprint disappears in non-proliferating cells, suggesting that the observed protein–DNA interaction is potentially involved in the process DNA replication [Dimitrova et al., 1996]. Taken together these results indicate that association with the nuclear matrix induces alteration in the chromatin structure of the origins representing more open chromatin configuration that could facilitate the establishment of initiation competent state of the mammalian origins of replication.

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