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Replication of the rat aldolase B locus differs between aldolase B-expressing and non-expressing cells

Satoru Miyagi[a,1], Yung-peng Zhao[a], Yasushi Saitoh[a], Katsuyuki Tamai[b], Ken-ichi Tsutsumi[a,*]

[a] *Cryobiosystem Research Center, Faculty of Agriculture, Iwate University, Ueda, Morioka, Iwate 020-8550, Japan*
[b] *Ina Laboratories, MBL Co., Ltd., Ina, Nagano 396-0002, Japan*

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Abstract We previously reported a rat chromosomal origin of DNA replication (oriA1) that encompassed the aldolase B (AldB) gene promoter. Here, we examined utilization of oriA1 in AldB-expressing and non-expressing cells. The results suggested the occurrence of mutually exclusive regulation between DNA replication and transcription. Nascent strand abundance as assayed by competitive polymerase chain reaction using bromodeoxyuridine-labeled nascent DNA indicated that oriA1 is not utilized in AldB-expressing cells, while it is fired in non-expressing cells. In the latter non-expressing cells, the replication fork seemed to slow at 20–22 kb downstream of oriA1.

Key words: Replication origin; Origin selection; Aldolase B gene; Promoter; Gene expression

1. Introduction

In higher eukaryotes, DNA in each chromosome replicates by multiple tandemly organized units called replicons, each of which has a defined origin of bidirectional replication. Firing of origins is temporally controlled during S phase [1–3], and occurs at developmentally defined origins [2,4–6]. In addition, it has been suggested that DNA replication is under cellular regulatory systems including those that govern transcription [4,5] and DNA repair [7–9].

Particularly, a causal relationship between initiation of DNA replication and transcriptional regulation might be of great interest in considering cell differentiation. If regulatory elements for transcription of certain genes are functionally connected with those for replication at certain initiation sites, then the positioning of fired origins may serve as an effector on transcription patterns in the chromosomal context. There may be a functional interaction between chromosomal positioning of origins and transcriptional regulation of nearby or even distant genes. Several related observations have already been reported. Replication of the rDNA region of *Xenopus* embryos initiates from randomly dispersed sites at an early stage of development, but with the beginning of transcription the initiation sites are restricted to the non-transcribed spacer region [10]. In addition, replication origins often encompass promoters or regulatory elements for transcription [11–14], and that initiation of replication is affected by such DNA elements [15,16]. In the case of the human β-globin locus, for example, mutant cells lacking a locus control region failed to replicate from the normal origin [16,17].

Despite these interesting observations, the mechanism operating in connecting transcription and replication is largely unknown.

We previously reported a chromosomal origin of DNA replication (termed oriA1) at the aldolase B (AldB) locus in AldB-non-expressing rat hepatoma cells, which encompassed the promoter for transcription [18]. The oriA1 region, when inserted into plasmids, confers autonomous replication in transfected cells [18–20], similar to that reported for human c-*myc* [14,21] and HSP70 genes [12]. Deletion and mutant analyses showed that the *cis*-elements within the proximal 200 bp promoter were necessarily required for autonomous replication [19,20], suggesting a functional correlation between replication initiation and transcription in vivo.

The present study was addressed to the functional correlation between firing of oriA1 and transcriptional regulation of the AldB gene in vivo. The results suggested that the organization of the replicon that encompasses the AldB gene differs between AldB-expressing and non-expressing rat hepatoma cells.

2. Materials and methods

2.1. Cell culture and BrdU labeling of nascent DNA strands

Rat hepatoma dRLh84 cells and FTO2B cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum and DMEM/Ham's F12 medium (1:1) supplemented with 10% fetal bovine serum, respectively, as reported previously [18]. For density labeling newly replicated DNA, growing cells were placed in a fresh medium containing 60 μM bromodeoxyuridine (BrdU, Sigma) and maintained at 37°C for the indicated time periods. In some cases, cells were arrested at the G1/S boundary by two rounds of cell cycle block using a medium containing 1.5 mM thymidine, and then released to synchronously enter into S phase, as previously reported [18]. Cell cycle phases were monitored by FACS (Becton Dickinson) analysis using propidium iodide-stained cells as reported [18,19].

2.2. Isolation of BrdU-labeled nascent DNA

After labeling with BrdU, cells were washed twice with phosphate-buffered saline, and treated with proteinase K. DNA was recovered as

*Corresponding author. Fax: (81)-19-621 6243.
E-mail address: kentsu@iwate-u.ac.jp (K.-i. Tsutsumi).

[1] Present adress: Division of Developmental Biology, Saitama Medical School, Research Center for Genomic Medicine, 1397-1 Yamane, Hidaka, Saitama 350-1241, Japan.

Abbreviations: PCR, polymerase chain reaction; BrdU, bromodeoxyuridine; AldB, aldolase B; HSP, heat-shock protein

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previously described [18]. DNA was denatured in 0.2 N NaOH, followed by neutralization and ethanol precipitation. BrdU-containing newly replicated DNA strands were then isolated by using anti-BrdU antibody and protein A+G agarose (MBL Co., Japan) (Fig. 1). BrdU–DNA preparations were 5′-phosphorylated with T4 polynucleotide kinase and ATP, and then digested with λ-exonuclease (see Fig. 1 for experimental procedure). By this digestion, nicked DNAs that were generated during preparation of DNA were eliminated while RNA-primed newly replicated DNA was not. The BrdU-labeled DNA was further size-fractionated by alkaline agarose gel electrophoresis. Short BrdU–DNAs ranging from 0.5 to 1.4 kb were removed from the gel, and used for PCR as reported by others [22]. Alternatively, BrdU–DNA was purified by CsCl density gradient ultracentrifugation as reported previously [18].

2.3. Quantitation of nascent DNA strands by competitive PCR analysis

Six sets of oligonucleotide primers (A–E) were designed to amplify the AldB gene and the flanking regions. Nucleotide sequences of the primers are given in Table 1, and locations of the amplified DNA regions were indicated below the restriction map shown in Fig. 2. Competitor DNAs used for primer sets A, B, C, D, and E were constructed from the corresponding PCR-amplified DNAs, by inserting the 186 bp *Pvu*II-*Eco*RV fragment of pBluescript SK$^-$ DNA (Stratagene) into *Eco*T14I, *Eco*T14I, *Eco*RV, *Nde*I and *Nhe*I sites of the respective amplified DNAs. For construction of the competitor DNA for primer set B′, a 98 bp long fragment from position −66 to −164 bp relative to the transcription start site of the AldB gene (+1) was deleted by using a commercially available in vitro mutagenesis kit (Takara, Japan). These competitor DNAs were subcloned into plasmid pBluescript SK. Appropriate amounts of the competitor plasmids were linearized by digestion with restriction enzymes, and subjected to PCR analysis. PCR was carried out under the standard condition in a volume of 20 μl. The cycle protocol was 94°C for 30 s, 53–55°C for 2 min and 72°C for 1–1.5 min for each cycle. Products were separated on an agarose gel, blotted, and hybridized with the appropriate probe fragments (Fig. 2). Quantitation of the amplified products was carried out by measurement of the autoradiographic bands (NIH Image 1.59b4, Paragon Concepts). Relative abundance of the target DNA was expressed as the amount of competitor DNA that gives an equal amount of the amplified target DNA as described by others [23].

3. Results and discussion

We previously identified an origin of DNA replication (oriA1, about 1.0 kb long) at the AldB gene region in rat hepatoma cell dRLh84, in which the gene expression is completely repressed [18]. Since the origin region encompassed a promoter for tissue-specific transcription of the AldB gene [18], we intended to determine whether the origin/promoter is similarly utilized in AldB-expressing and non-expressing cells. For this purpose, we chose rat hepatoma FTO2B cells [24] as a typical cell line that actively expresses the AldB gene (Fig. 3). Delimitation of replication origins on the genome was carried out by analysis of the relative abundance of

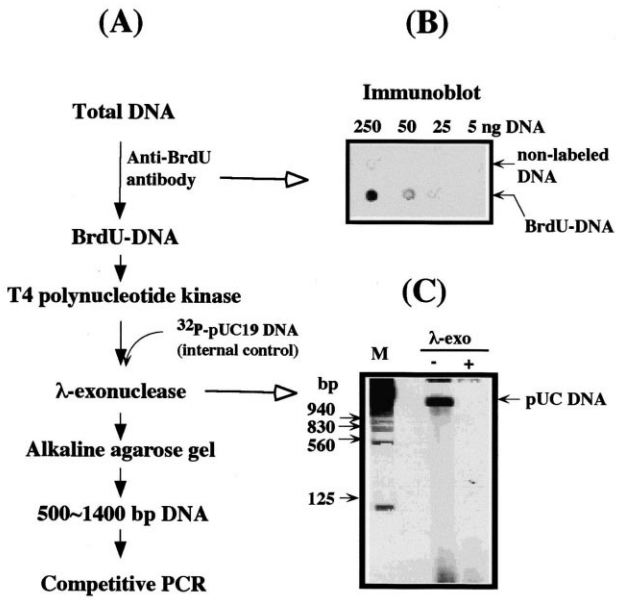

Fig. 1. Preparation of BrdU-labeled short nascent strands. A: Flow diagram of nascent strand preparation. B: Immunoblot using anti-BrdU antibody showing specificity of the antibody. Indicated amounts of BrdU-labeled DNA and non-labeled DNA were spotted onto a nylon membrane and incubated with anti-BrdU antibody. DNA-bound antibodies were detected using the ECL Western blotting analysis system (Amersham Pharmacia Biotech). BrdU-labeled DNA was purified by CsCl density gradient centrifugation from dRLh84 cells labeled for 48 h with BrdU. C: λ-Exonuclease digestion of ^{32}P-labeled pUC19 DNA that was included in the BrdU–DNA preparation as an internal control. An aliquot of the digested sample was subjected to polyacrylamide gel electrophoresis and subjected to autoradiography.

BrdU-labeled short nascent DNA chains using randomly cultured cells; the procedure has been successfully employed for several other origins [23].

Randomly cultured FTO2B and dRLh84 cells were pulse-labeled with BrdU (60 μM) for 15 min. BrdU-containing DNA fragments were prepared by using anti-BrdU antibody (MBL Co., Japan). Nicked DNA which was possibly generated during DNA preparation was eliminated by digestion with λ-exonuclease, and then remaining RNA-primed BrdU–DNA was size-fractionated by agarose gel electrophoresis as described in Section 2. Fig. 1B,C shows specificity of the antibody used in this experiment, and λ-exonuclease digestion of 5′-^{32}P-labeled pUC DNA that is included as an internal control, respectively. The results confirmed that the

Table 1
Oligonucleotide primers used for PCR

Primer set		Nucleotide sequence (from 5′ to 3′)	Length of PCR product (bp)
A	sense	CTTCATGGAGTCATTCATGG	490
	antisense	ATCCACATGCAGCAACTTGG	
B	sense	TACTTACACAGCCAAGGAAGA	500
	antisense	TGGACATGTAGCTTAGTGTA	
B′	sense	TTATAGTCTCCTCACCTGTA	250
	antisense	GTCAAATAGGATGGATCTGC	
C	sense	GAACTGTTCCTGCAGCTGTG	420
	antisense	GCAGATACCTGCAGTGAGAG	
D	sense	AACAACCTGATGACTGTTCC	470
	antisense	TGGACATGTAGCTTAGTGTA	
E	sense	AGAATCCAAACTTAGTTGTCA	490
	antisense	GTGAGTAGCCTTAACTTCTG	

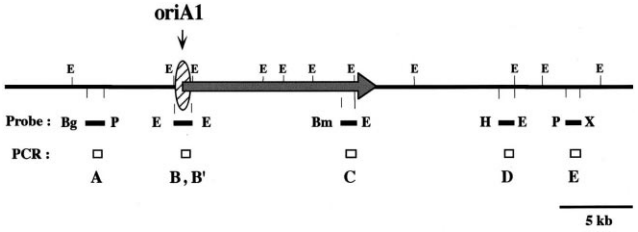

Fig. 2. Structure of the rat AldB gene. The arrow represents the AldB gene, and the hatched circle indicates the origin of bidirectional replication (oriA1) in rat hepatoma dRLh84 cells [18]. Vertical lines represent recognition sites for restriction enzymes. E, *Eco*RI; Bg, *Bgl*II; P, *Pst*I; Bm, *Bam*HI; X, *Xho*I. Boxes A–E show positions of primer sets A–E (their nucleotide sequences are listed in Table 1) for PCR and DNA regions amplified. Horizontal bars below the map represent the DNA fragments used as probes for hybridization experiments.

antibody specifically recognizes BrdU–DNA, and that 5′-phosphorylated DNA is completely digested by λ-exonuclease.

To determine the relative abundance of newly replicated short DNA fragments derived from the AldB gene region, the BrdU-containing short nascent DNA (0.5–1.4 kb long) was subjected to competitive PCR analysis. Fig. 4A shows the products of PCR using fixed amounts of the short BrdU-labeled DNA in the presence of the competitors. Relative abundance of the target DNA in each sample was expressed as the amount of competitor DNA in the PCR mixture that gave an equal amount of the amplified target DNAs (Fig. 4B). In dRLh84 cells, the relative abundance was highest in region B as compared to those in regions A, C, D, and E, indicating the B region to be the replication initiation region. This confirms our previous results obtained by different experimental approaches [18]. However, short nascent strands derived from the B region in FTO2B cells had a significantly lower abundance as compared to that in dRLh84 cells (less than one tenth). In addition, there were no significant differ-

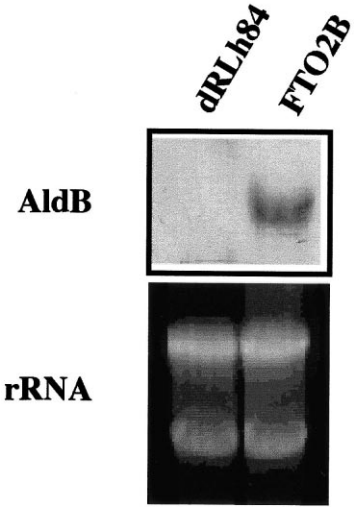

Fig. 3. Expression of the AldB gene in rat hepatoma cells dRLh84 and FTO2B. Total RNA (30 μg) prepared from dRLh84 and FTO2B cells was separated on an agarose gel, blotted and hybridized with ^{32}P-labeled AldB cDNA. The lower panel shows ethidium bromide staining of RNA to show equal amounts of loading.

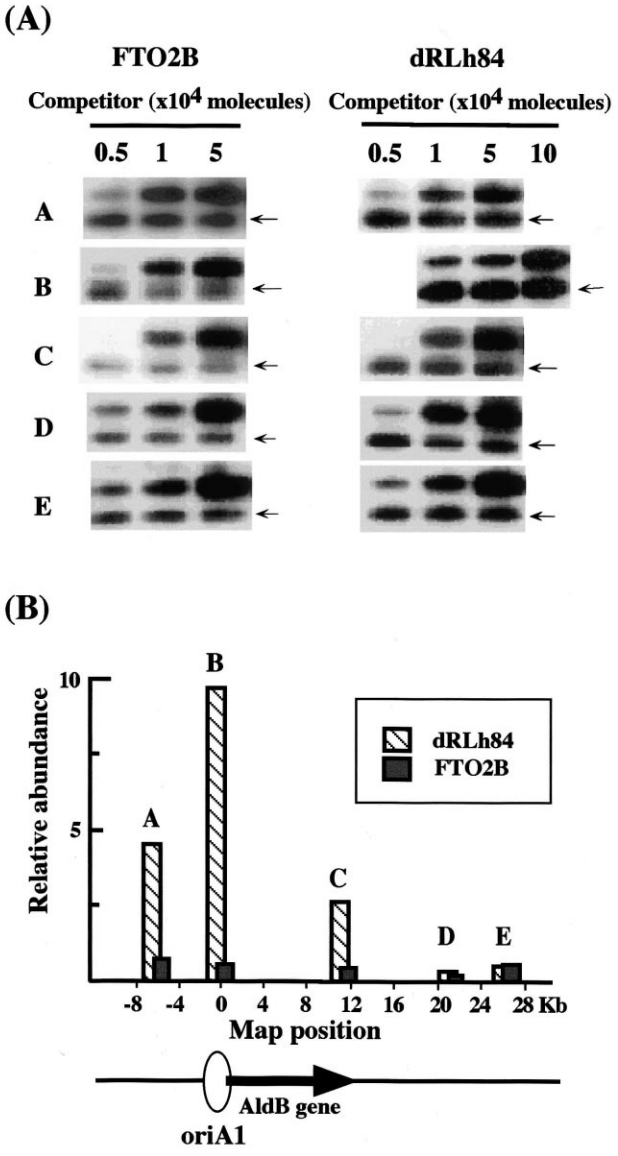

Fig. 4. Competitive PCR for determination of relative abundance of newly replicated DNA in AldB-expressing (FTO2B) and non-expressing (dRLh84) cells. A: Products of competitive PCR using BrdU-containing newly replicated DNA (0.5–1.4 kb long) as a template. Cells were labeled with BrdU for 15 min, and newly replicated BrdU-containing short DNA was prepared (Fig. 1, see also Section 2). Equal amounts of BrdU-labeled DNA were then subjected to competitive PCR using primer sets A–E listed in Table 1 (for their positions see Fig. 2). Amounts of competitor DNA added in the PCR reaction mixtures are 5×10^3–1×10^5 molecules as indicated at the top of the figure. The PCR-amplified DNAs were separated on an agarose gel, blotted onto a nylon membrane and hybridized with the DNA fragments corresponding to the amplified regions (see Fig. 2). The target DNAs are indicated by arrows. B: Relative abundance of BrdU-containing newly replicated DNAs corresponding to regions A–E, as expressed by the amounts of competitor DNA in the PCR mixture that gave equimolar amounts of the amplified competitor and the target DNAs based on calculation from the intensities of bands. Map positions are indicated as the distance from the transcription start site of the AldB gene in kb. The horizontal arrow represents the transcription unit of AldB gene.

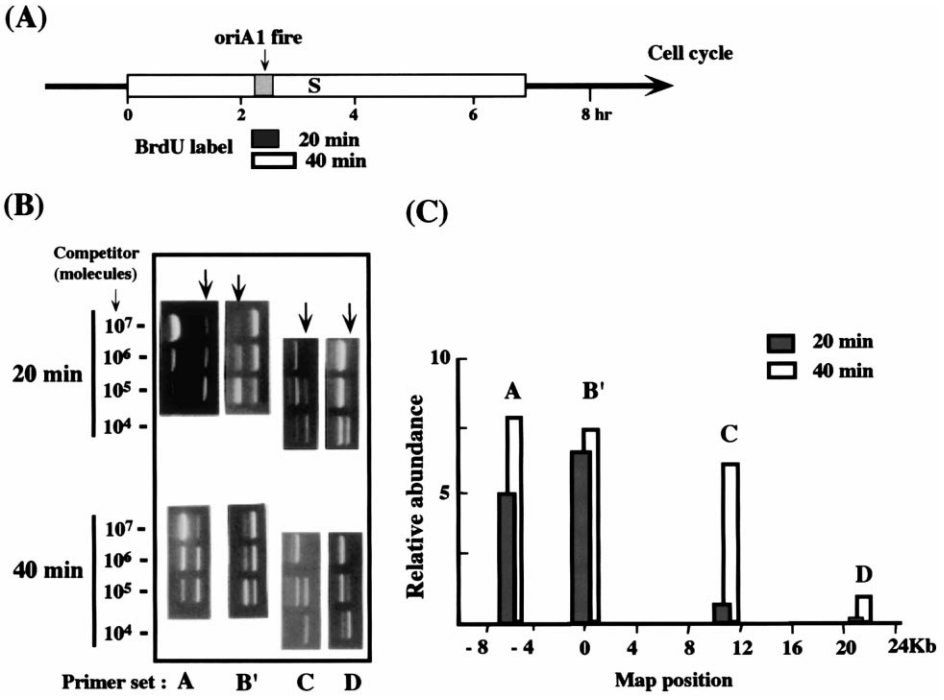

Fig. 5. Replication delays at specific chromosomal DNA regions in dRLh84 cells. A: Time schedule for pulse labeling with BrdU. Synchronously cultured dRLh84 cells were pulsed-labeled with BrdU for 20 min (filled box) from the time points 140 to 160 min after entering S phase, or 40 min (open box) from the time points 140 to 180 min. Box represents S phase, and time zero indicates release from the arrest at the G1/S boundary. Firing of oriA1 occurs around 150 min [18]. B: Competitive PCR analysis for determination of relative abundance of the BrdU-labeled DNA strands. BrdU-labeled single-stranded DNA was isolated by alkaline CsCl density gradient centrifugation [18]. The BrdU–DNA was amplified in the presence of increasing amounts of competitor DNA as described in Fig. 4, and the amplified DNA was separated on an agarose gel. Arrows indicate target DNA in each PCR product. C: Relative abundance of newly replicated DNA regions. Filled and open boxes represent amplified DNAs from 20 and 40 min-labeled DNAs, respectively.

ences in the relative abundance throughout the AldB gene region in FTO2B cells; the abundance was equally low from the A to the E region. Labeling for a longer time (e.g. 120 min) with BrdU resulted in an increase in relative abundances of nascent strands from region A to E (data not shown). Therefore, it is considered that the promoter region in AldB-expressing FTO2B cells was not utilized as an origin of bidirectional replication.

We next examined replication timing of regions A–D in and around the AldB gene, by similar PCR experiments using BrdU-labeled DNAs from synchronously cultured dRLh84 cells. Cells were arrested at the G1/S boundary by double thymidine block, and synchrony of the cell cycle phase after release from the arrest was monitored by FACS analysis as reported previously (data not shown) [18,19]. Since oriA1 in dRLh84 cells has been shown to fire at around 150 min after release from the arrest under our culture condition [18], BrdU labeling was conducted to start at 140 min after entering S phase for either 20 or 40 min (see Fig. 5A). After labeling, BrdU-containing single-stranded DNA was prepared by alkaline CsCl isopycnic ultracentrifugation [18], and subjected to competitive PCR as in Fig. 4. Analysis using cells labeled for 20 min (from time point 140 to 160 min) revealed that product from region D was more than 10 times less abundant as compared to those from regions A and B (Fig. 5B). Continuous labeling for an additional 20 min (40 min label from 140 min to 180 min) increased the abundance of region C to a level similar to those of regions A and B, whereas that of region D remained low (Fig. 5C). Labeling for a longer time period (120 min) increased the amount of newly replicated D region DNA to a level similar to that of region B (data not shown). Thus, region D apparently replicated late.

In this paper, we have described the mode of replication of the rat AldB gene region in AldB-expressing and non-expressing cells. Initiation of DNA replication from the AldB origin, oriA1, was not common to growing cells but is regulated cell type-specifically. Replication did not initiate from oriA1 in FTO2B cells in which the AldB gene is actively transcribed. Thus, mutually exclusive regulation between DNA replication and transcription might exist, i.e. firing of oriA1 represses the AldB gene transcription or vice versa. Possibly, alternative architecture of the protein–DNA complexes at the oriA1/promoter region might play a crucial role. Indeed, binding of nuclear factors to the promoter elements of the AldB gene differs between AldB-expressing and non-expressing cells. Binding of a set of specific factors confers tissue- and development-specific transcription [25–31], while in AldB-non-expressing rapidly dividing cells such as dRLh84 and fetal liver cells, a different set of factors binds to this promoter region [19,31–33].

Progression of the replication fork through the AldB gene region after firing of oriA1 was not continuous but seemed to slow down or pause at around the D region (20–22 kb downstream of oriA1) in dRLh84 cells, since this region replicated late in synchronously cultured cells. Such replication fork pause or arrest has been reported for rDNA regions in human [34], mouse [35], yeast *Saccharomyces cerevisiae* [36–38] and plant *Pisum sativum* [39], and for histone gene repeats in *Drosophila* [40]. It has been thought to be a mechanism to prevent collision between transcribing RNA polymerase complex and

replication forks moving in a direction opposite to transcription. However, this situation may not be true in the case of the AldB gene region in dRLh84 cells, because the gene is completely inactivated. Even if a small extent of transcription occurs, transcription and replication are in the same direction. In addition, no transcription unit was detected in the vicinity of the putative pause region (data not shown). Physiological roles are unknown at present.

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