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Initiation zone of DNA replication at the aldolase B locus encompasses transcription promoter region

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ABSTRACT

Aldolase B (AldB) gene is one of the liver-specific genes, which is activated in the fetal stage. As a first step to investigate the functional relationship between transcription and DNA replication, we intended to determine the initiation zone of replication nearest to the AldB gene region. BrdU-labeled nascent DNA was obtained from G1/S arrested hepatoma cells at various times after entering S phase. Hybridization of the newly synthesized, BrdU-labeled DNA with probes corresponding to regions spanning about 26 Kb, revealed that replication zone locates within the AldB gene region. This result, together with the result of hybridization of nascent DNA obtained by alkaline sucrose density-gradient centrifugation, suggested that the initiation zone is located within a more defined region (about 1.0 Kb) containing AldB promoter. In the predicted initiation zone, a purine-rich element which shows high homology to known mammalian origin sequences and other replication components are found. Further, autonomously replicating activity of this initiation zone was examined by DNA transfection. The results showed that the predicted initiation zone confers replication initiation in Cos-1 cells.

INTRODUCTION

Previous studies have demonstrated that replication of specific genes does not occur randomly throughout the genome. Replication begins at specific initiation sites, which are activated in a temporal and spatial order during S phase (1–3). Generally, replication timing of tissue-specific genes are under developmental control; most transcriptionally activated genes are replicated early in S phase, while quiescent genes are replicated later (4,5). In simple genomes, some *cis*-elements for transcription act as auxiliary elements for initiation of DNA replication, and in some complex genes, DNA replication may be initiated from different origins in different cell types (6,7). Several factors concerning DNA replication have been reported to be involved in transcription regulation (8,9). CTF/NF-1 is one such mammalian DNA-binding protein, which is known to activate both transcription and initiation of replication of viral DNA (10). It has been reported that *cis*-acting elements for transcription may play a direct role in determining the temporal order of activation of replication origins during S phase (11–13). Conversely, recent studies indicate that transcriptional reaction inhibits replication (14). Stillman *et al.* isolated a multiprotein complex called the origin recognition complex (ORC) which binds to an essential element of autonomously replicating sequence (ARS) in *S.cerevisiae* chromosome. ORC is a strong candidate for the eukaryotic initiator protein required for DNA replication (15) and is also shown to be involved in transcriptional repression (16–18). These observations strongly suggest a functional relationship between transcription and DNA replication. However, little is known about such a relationship. Elucidation on how replication affects transcription and what mediates the two cellular reactions will lead to know the mechanism operating in the switch from proliferation to differentiation of cells or *vice versa.*

Rat aldolase B (AldB), one of the three isozymes (A, B and C) of fructose-1,6- bisphosphate aldolase, is selectively expressed in the liver of adult animals and certain differentiated hepatoma cells, but repressed in the fetal liver and dedifferentiated hepatoma cells (19). Liver-specific expression of the gene is controlled by *cis*-acting regulatory elements in the proximal 200 bp promoter, to which a number of transcription factors interact (20–23). Our interest lies on how and why transcription of the AldB gene is repressed in the fast-growing cells, i.e., fetal livers and hepatoma cells. As a first step to understand the functional and positional relationship between DNA replication and transcription of the AldB locus, we determined the initiation zone of replication nearest to the AldB gene.

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MATERIALS AND METHODS

Cells and culture conditions

Rat hepatoma cells (dRLh84) and Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn bovine serum and 10% fetal bovine serum, respectively, supplemented with 4 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 100 μg/ml kanamycin. The cultures were maintained in humidified atmosphere containing 5% CO_2 at 37°C.

Cell synchronization

Growth cycle of the hepatoma cells was synchronized at G1/S phase by two rounds of thymidine block (24) as follows. Cells (5×10^5 cells per 100 mm dish) were grown for 36 h. At logarithmic growth phase, thymidine was added to a final concentration of 1.5 mM to arrest cell cycle at G1/S boundary. After 14 h, the cells were washed three times with phosphate-buffered saline (PBS) at 37°C, and were added with fresh medium. The cells were grown for an additional 8 h and thereafter, they were treated with 1.5 mM thymidine for 14 h as above. After withdrawal of thymidine, the cells were allowed to progress synchronously through S-phase.

BrdU density-labeling and isolation of DNA

To density-label nascent DNA, bromodeoxyuridine (BrdU) (Sigma Chemical Co.) was added to the synchronously cultured cells to a final concentration of 10 μM immediately after releasing from the second thymidine block. At each experimental time point, the cells were washed with ice-cold PBS(−) and harvested. Following 100 μg/ml proteinase K treatment for 12 h, DNA was extracted three times with phenol and precipitated with ethanol. The DNA was separated by isopycnic ultracentrifugation in CsCl at 100 000×g at 20°C for 72 h. After centrifugation, 150 μl fractions were collected from the bottom of the tubes. Each fraction was dialyzed against 10 mM Tris−HCl (pH 7.4) containing 1 mM EDTA to remove CsCl.

Alkaline sucrose density-gradient centrifugation of nascent DNA

Cells were harvested and suspended in 10 mM Tris−HCl (pH 7.4) containing 0.15 M NaCl and 1 mM EDTA. The cell suspension was directly loaded onto lysis solution (0.45 M NaOH, containing 0.55 M NaCl, 10 mM EDTA and 1% sodium N-lauroyl sarcosinate), which was overlayed on a solution consisting of 5−20% linear sucrose gradient containing 0.1 M NaOH, 0.9 M NaCl and 50 mM EDTA. The lysed cells were centrifuged for 24 h at 4 °C at 77 000×g. After centrifugation, DNAs were fractionated into three portions from the bottom to the top. Each fraction was neutralized by adding 1 N HCl and precipitated with ethanol. The DNA samples were separated on an alkaline agarose gel in 30 mM NaOH containing 2 mM EDTA and 50 mM NaCl at 1.5 V/cm (25).

Probes and hybridization conditions

DNA fragments corresponding to the AldB gene region were purified by agarose gel electrophoresis and used as probes. Hybridization was carried out at 65°C in 0.25 M Na_2HPO_4 (pH 7.4) containing 1 mM EDTA and 7% SDS as described by Church and Gilbert (26).

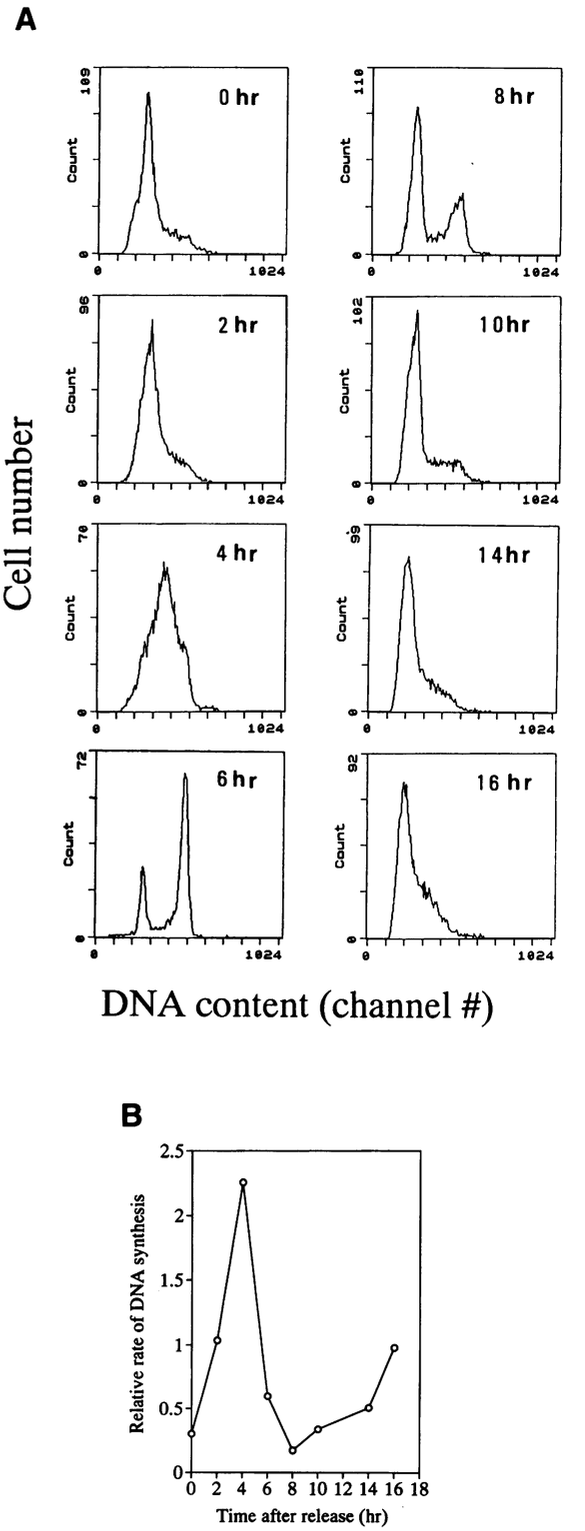

Figure 1. Cell cycle analysis of the synchronously cultured rat hepatoma cells. **(A)** Growth of cells (dRLh84) was arrested at G1/S boundary by double thymidine block (see Materials and Methods). At the indicated time points after release from the cell cycle block, the cells were stained with propidium iodide and subjected to flow cytometry for determination of DNA content. **(B)** Relative rate of DNA synthesis. Synchronously cultured cells at various times were pulse-labeled with ^{3}H-thymidine for 20 min. Values represent relative rate of ^{3}H-thymidine incorporation per min per cell.

Cell cycle analysis

Synchronously cultured cells were harvested at various experimental time points. The cells were trypsinized, washed in PBS(−) and fixed in cold 70% ethanol. After washing with PBS(−) and RNase treatment, approximately 6×10^6 cells were stained with propidium iodide (PI; Sigma) and examined for DNA content and ploidy by using an EPICS Elite cell sorter (Coulter, USA).

In vivo DNA synthesis

Synchronized cells were pulse-labeled with 5 μCi/ml of [methyl-^{3}H]thymidine (20 Ci/mmol, Amersham Corp.) for 20 min at 37°C. Cells were washed twice with ice-cold PBS(−) containing 1 mM thymidine and pelleted by centrifugation. The cell pellet was solubilized in 1 ml of 0.2 N NaOH. The solubilized cells were neutralized with 1 N HCl, and precipitated with ice-cold 5% trichloroacetic acid (TCA). The pellet was rinsed with 5% TCA and then with ethanol, dried and measured for radioactivity in a liquid scintillation counter.

Transfection and *in vivo* replication assay

The plasmid pBOR0.94 was constructed by ligating a 0.94 Kb fragment extending from −675 to +263 bp (relative to the transcription start site) of the AldB gene to the *Hin*dIII site of plasmid pUC19. Ten μg of plasmid pBOR0.94 or pUC19 per 100 mm dish was transfected into Cos-1 cells using calcium phosphate coprecipitation method (27). After 12 h of post-transfection, the medium was changed and BrdU was added to a final concentration of 20 μM. After 48 h at 37°C, cells were washed twice with PBS(−) and DNA was extracted according to the method described by Hirt (28). The DNA was digested with *Hin*dIII, and BrdU-containing DNA was separated by CsCl isopycnic centrifugation. Fractions were collected from the bottom and electrophoresed in an 1% agarose gel. DNA was transferred onto a nylon membrane and hybridized with ^{32}P-labeled linearized pUC19 DNA.

RESULTS

Replication of the AldB locus occurs during mid S-phase and initiates near or within the gene region

DNA replication in mammalian chromosomes is initiated bidirectionally at specific sites. We intended to know the replication origin nearest to the AldB locus by detecting direction of replicating DNA strands. In order to obtain a large population of replication forks in the replicating zone, we thought it would be necessary to synchronize the cells at an early S phase. Thymidine, an inhibitor of ribonucleoside diphosphate reductase which inhibits DNA synthesis, was used to arrest rat hepatoma cells (dRLh84) at the G1/S boundary. The population of cells at specific cell cycle phase was measured by using a flow cytometry after withdrawal of thymidine (Fig. 1A). DNA synthesis was also measured by [^{3}H]thymidine incorporation (Fig. 1B). It is apparent from the figure that the cells synchronously progress through the cell cycle after releasing cells from thymidine block. The synthesis of DNA began immediately after the withdrawal of thymidine and reached a maximal level at about 4 h. DNA synthesis was markedly decreased at 6 h after release from thymidine block (Fig. 1B). Thus, the S-phase starts shortly after the withdrawal of thymidine and ends within ~6−7 h. Measurement of the average DNA content of the cells using flow cytometry confirmed this result (Fig. 1A). These data show that a high percentage of synchronous cell population can be prepared by this procedure.

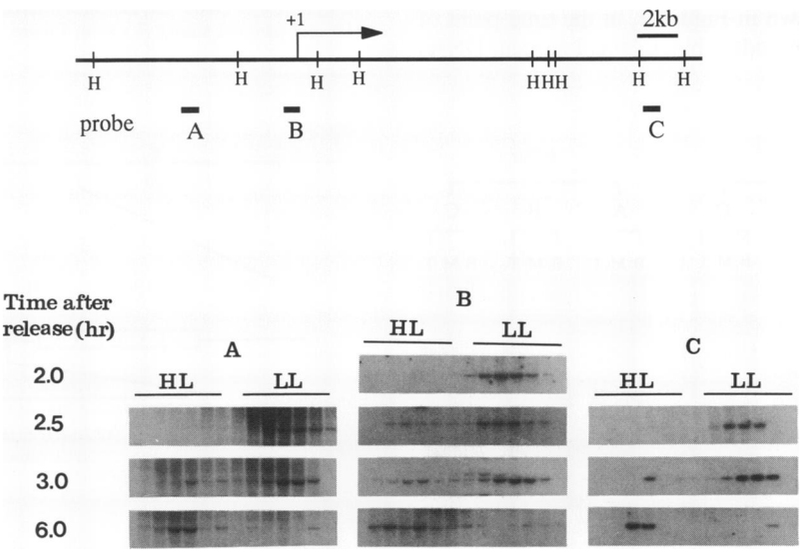

Figure 2. Southern blot analysis of BrdU-containing nascent DNA. Restriction map of the AldB gene region showing fragments used as probes for hybridization is shown in the upper panel. H, *Hin*dIII. Numbers indicate positions relative to the transcription start site. Lower panel shows detection of newly synthesized, BrdU-containing DNA. Cells were labeled with BrdU for 2, 2.5, 3 and 6 h (indicated on the left) after release from cell cycle block (see Materials and Methods). DNAs were then prepared, digested with *Hin*dIII and subjected to CsCl isopycnic ultracentrifugation. Each fraction from the bottom of the tube was run on an agarose gel, blotted and hybridized with probes A, B and C indicated on top of the autoradiograms. HL indicates heteroduplex consisting of BrdU-containing heavy chain (H) and parental light chain (L). LL indicates heteroduplex consisting of parental light chains.

To determine when and where initiation of DNA replication near the AldB gene occurs, the G1/S arrested cells were then cultured in a fresh medium containing BrdU, and forced to enter S phase (see Materials and Methods). After various time periods, DNA was extracted and heteroduplex containing BrdU-labeled DNA strand was separated by neutral CsCl isopycnic ultracentrifugation. Fractions were collected from the bottom of the tube, and analyzed by 1.0% agarose gel electrophoresis. After blotting, DNA in each fraction was hybridized with various probes corresponding to the AldB gene region. The results are shown in Figure 2. At 2 h after release of dRLh84 cells from the G1/S boundary, no hybridization of BrdU-containing nascent DNA (heteroduplex designated as HL, H; BrdU-containing heavy chain, L; light chain) with probes A, B and C (see map in Fig. 2) were observed. However, at the time points of 2.5 h, hybridization signal of BrdU-containing nascent DNA for the probe B was observed, whereas no apparent signal was observed for the probes A and C. At 3 and 6 h after entering S phase, nascent DNAs corresponding to all sites A, B and C were detected. Hybridization signals of non-replicated DNA (LL) for probes A, B and C almost disappeared at 6 h after entering S phase, indicating that replication of the AldB gene region in most cells had already finished. These results strongly suggest that the initiation zone of the AldB gene locates between regions A and C, possibly near region B.

Delimitation of the initiation zone

To know the initiation zone more precisely, newly-replicated, single-stranded DNA chains were directly fractionated from the synchronized cells by 5–20% alkaline sucrose-density gradient centrifugation (see Materials and Methods). Through this process, relatively small fragments centered around the origin could be isolated in a low-molecular-weight fraction. After centrifugation, DNAs were fractionated into three portions from the bottom of the tube; namely, bottom (B), middle (M) and top (T). These DNAs were separated on an agarose gel, and hybridized with probes A, B and C. As shown in Figure 3, at the time point of 2.5 h after releasing from thymidine block, small nascent DNAs which hybridized with probe B were detected in the middle and top fractions, the minimum length of which is about 0.5 Kb. However, no hybridization was observed using either probes A or C, except the high-molecular-weight DNA (fractions B and M). At 6 h, all probes A, B and C hybridized with only high molecular weight DNA, indicating there is no replicating origin near the AldB locus at this time point.

To ascertain the presence of the sequence corresponding to the probe B region, the low-molecular-weight DNA fraction (indicated by bracket in Fig. 3) obtained from cells at 2.5 h after entering S phase was subjected to PCR (data not shown). Primers were designed to amplify regions corresponding to probes A, B and C. The DNA fragment corresponding to region B was amplified efficiently, but others were not. Taken together, these results are consistent with those using BrdU-labeled DNA described as above (Fig. 2). Considering the size of probe B and the size of the smallest single-stranded DNA that hybridized with

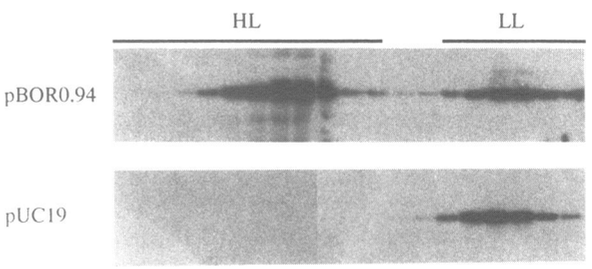

Figure 4. *In vivo* replication of plasmid containing predicted replication initiation region. A plasmid containing AldB gene fragment extending from −675 to +263 bp (pBOR0.94, upper panel) or a plasmid pUC19 (lower panel) was transfected into Cos-1 cells. After growing the cells for 48 h in the presence of BrdU in culture, DNAs were prepared, digested with *Hin*dIII and fractionated by CsCl isopycnic ultracentrifugation as described in the legend to Figure 2. DNA in each fraction was separated on an agarose gel, blotted and hybridized with the ^{32}P-labeled pUC19 DNA. HL indicates heteroduplex containing BrdU-labeled strand (H) and parental strand (L) as in Figure 2.

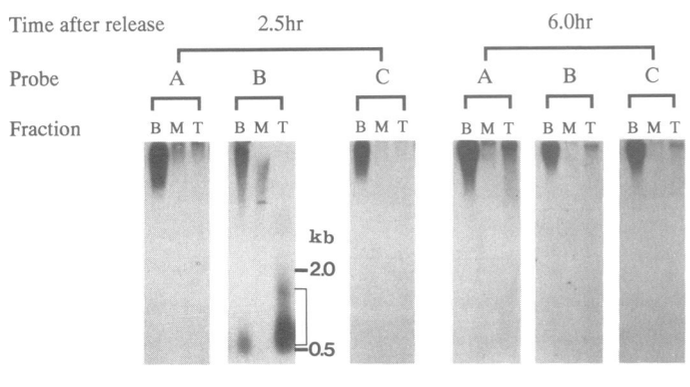

Figure 3. Detection of replicating short fragments by alkaline sucrose density-gradient centrifugation. Cells cultured for 2.5 h (left) and 6 h (right) after release from the cell cycle block, were directly lysed in alkaline solution and fractionated by alkaline sucrose density-gradient centrifugation as described in Materials and Methods. Three fractions (bottom, B; middle, M and top, T) were collected from the bottom of the tube, and subjected to agarose gel electrophoresis. After blotting, DNAs were hybridized with probes A, B and C shown in Figure 2. Bracket in the figure indicates low-molecular-weight DNA used for PCR (see text).

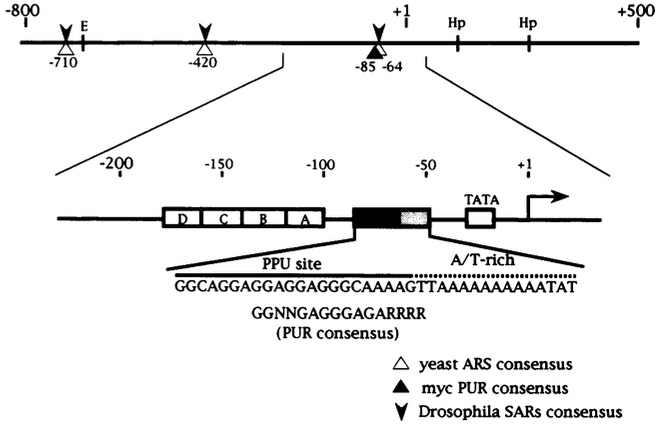

Figure 5. Structural features of the predicted initiation region. Restriction map of AldB gene region from −800 to +500 bp, which include replication origin, is shown. E, *Eco*RI; Hp, *Hpa*II. Boxes A–D and TATA box represent elements for transcription (20–23). Sequences having high homology to yeast ARS (open triangles), PUR consensus found in myc origin (filled triangle) and scaffold-associated regions (SARs, arrow heads) in *Drosophila* are shown.

it (Fig. 3), DNA replication of AldB gene region initiates from within less than 1.3 Kb region encompassing the probe B region.

Autonomous replication in Cos-1 cells

Since it was demonstrated that several mammalian chromosomal origins possess activities to replicate autonomously (29), we examined whether AldB gene origin promotes replication in Cos-1 cells. For this purpose, a 938 bp fragment extending from −675 to +263 was inserted into *Hin*dIII site of pUC19 plasmid (designated as pBOR0.94), and transfected into Cos-1 cells. The transfected cells were cultured in the presence of BrdU for 2 days and DNA was extracted by the method described by Hirt (28). BrdU-containing newly-synthesized DNA was fractionated by CsCl density-gradient centrifugation after digestion with *Hin*dIII. Each DNA fraction from the bottom was separated on an agarose gel and hybridized sequentially with ^{32}P-labelled pUC19 fragment and 938 bp fragment (data not shown) (see Materials and Methods). Figure 4 (upper panel) clearly shows that newly-synthesized DNA fraction (HL region, H; heavy chain containing BrdU, L; light chain of parent DNA) hybridized with pUC19 probe. In contrast, when pUC19 plasmid with no DNA insert was introduced into Cos-1 cells, no signals of DNA replication were found (Fig. 4, lower panel). The 0.94 Kb fragment directed replication reproducibly by several-fold or more as compared to the control.

Structural feature of the predicted initiation region

Figure 5 shows the structural features of AldB promoter region, a part of the predicted replication initiation region as mentioned above. Boxes A−D represent *cis*-elements required for regulation of liver-specific transcription (20−23). In addition to these, a unique sequence (designated as PPU) enriched in purine bases is present. This sequence is flanked by A/T-rich region, and shows a high degree of homology with PUR consensus that is commonly seen in origin sequences in several eukaryotic genomes (see Discussion). Consensus sequences for SAR (scaffold-associated region) were also found in the promoter region. Chromosomal DNA attaches to the nuclear scaffold *via* this DNA sequence, where replication is proposed to occur (32).

DISCUSSION

In the present study, we identified a putative initiation region of DNA replication near the AldB gene in rat chromosome, based on the analysis of Southern blot hybridization of BrdU-labeled nascent DNA prepared from synchronously cultured cells at S phase. The origin region was confirmed by hybridization with various probes and by PCR amplification of newly-synthesized, low-molecular-weight DNA. The predicted origin region locates within as large as 1.3 Kb and encompasses promoter region for transcription. To further confirm this, a 0.94 Kb fragment corresponding to the predicted origin region was inserted into plasmid and introduced into Cos-1 cells. This plasmid was found to autonomously replicate (Fig. 4). Thus, the 0.94 Kb fragment contains a basal components that serve for DNA replication.

Within this very region contains a unique sequence motif consisting of a 22-mer of purine-rich element (designated as PPU) which has high homology (13 out of 16 bp) to the PUR consensus sequence, a purine rich element found in the origins of yeast, human β-globin, human c-myc and hamster DHFR genes (13,30,31). In HeLa cells, the PUR element binds to a 28 kDa protein which is believed to act as a sequence-specific helix-destabilizing factor for initiation of DNA replication (30). The PPU sequece in the AldB gene is flanked by a 15 mer of A/T-rich sequence. A/T-rich element is also one component of *ori* core sequence in origin region, which is required as a DNA unwinding element for initiation of replication in yeast and animal viruses (32,33). Coexistence of a PUR consensus sequence in the vicinity of an A/T rich element in the predicted origin region at the AldB locus matches the structural features described above. It has also been suggested that, in some cases, *cis*-elements for transcription act as auxiliary elements for replication (6). Within the predicted initiation region of AldB replication locates at least three important *cis*-elements for liver-specific transcription (see Fig. 5, refs 20−23). Whether these elements are involved in replication events or not, is of great interest.

Unlike simple genomes such as those of viruses, the complex genomes of eukaryotes do not replicate from a single origin and so have multiple origins (34). Although previous studies suggested that DNA replication initiates from specific origins, it is yet uncertain whether a single defined origin or multiple tandemly arrayed origins constitute a large replication unit. Nascent strand analyses demonstrated that replication of the DHFR locus initiates from within a few kilobase pairs in chromosome of CHO cells (35,36). Polymerase chain reaction (PCR) (37) and analysis of Okazaki fragments (38) narrowed the initiation site to a 2.5 and 0.45 Kb fragments, respectively. However, the results obtained from two-dimensional (2D) gel electrophoretic mapping techniques suggested that initiation could occur at multiple sites within a broad initiation zone of 55 Kb (39). In support to this observation, *in vivo* replication assay revealed that human genomic DNA sequences required to direct replication have been reported to be greater than 10 Kb (40). Further, the autonomous replication seems to initiate from multiple sites without sequence specificity (40,41). Our results appear to contradict these previous reports, in that replication of the AldB gene initiates sequence specifically from a defined region, but not from multiple sites in a broad zone. Most recently, Kitsberg *et al.* located a 2.0 Kb initiation zone near the β-globin locus in human cells. When this initiation zone was deleted, the DNA replication initiated from far upstream, but did not occur near the position of the presumptive origin relative to the β-globin locus (13). Our results are consistent with this observation, indicating that DNA replication depends primarily on some defined origins which are more efficient than other potential origins.

Replication timing of human β-globin gene domain is under developmental control but the initiation site is topographically fixed (13). In contrast, active and inactive chicken histone H5 genes are replicated from different origins (7). The reasons and meanings for these differences remain unknown, but they should be further explored with regard to their implications for replication and transcription. It would be very interesting and important to know the relationship among replication time, site of replication origin, and potential of transcription of the AldB gene region.

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