

# Initiation of DNA replication at the rat aldolase B locus

—An overlapping set of DNA elements regulates transcription and replication?—

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## Summary

**In higher eukaryotes, DNA replication initiates at multiple sites on each chromosome. Positioning and firing of the replication origins are not fixed, but different and selected origins may initiate at different times in a single cell cycle of particular cells through a range of complex mechanisms controlling, for example, cell differentiation. The origin region at the rat aldolase B locus (ori A1) has been found to encompass the promoter which governs liver-specific transcription. Ori A1 is, thus, thought to be a suitable target in investigating causal relationships among those under control of cell differentiation, i.e., firing or silencing of the origin, cell type-specific regulation of transcription, and positioning of nearby origins. In this article, we summarize our approach to elucidate such relationships. We describe sequence-dependent replication from ori A1, overlapping of essential regions required for replication and transcription, cell cycle-regulated binding of factors to the essential region, and then chromosomal state of the ori A1 region in the nucleus.**

## I. Introduction

Initiation of DNA replication is one key control point in a process of cell cycle progression. In eukaryotic cells, each chromosome contains multiple replication origins which are activated in a stringently controlled temporal and spatial order during S phase. In addition, not all origins are fired for genome duplication in a single cell cycle, some origins are generally used while others are not (DePamphilis, 1993a; Coverley and Laskey, 1994; Hamlin et al., 1995; Stillman, 1996). Which origins are selectively used and when they are activated are, however, not fully understood. The selection and positioning of origins on the chromosomes might be controlled by multiple regulatory processes such as developmental program, transcription activity of nearby genes, and firing of nearby origins (James and Leffak, 1986; Wolffe and Brown, 1988; Trempe et al., 1988; Leffak and James, 1989; Fangman and Brewer, 1992).

Evidences have been accumulating that cis-elements for transcription promote replication activity as well (for

review see DePamphilis, 1993b). For example, transcription factors AP1 (Guo and DePamphilis, 1992), NF1 (Mul and Van der Vliet, 1992), Oct1 (O'Neill et al., 1988) and c-Jun (Ito, Ko et al., 1996) strongly stimulate virus DNA replication. In cellular chromosomes, origin region or regulatory region for DNA replication often encompasses transcriptional promoter or contains cis-elements for transcription (Vassilev and Johnson, 1990; Ariizumi et al., 1993; Taira et al., 1994; Tasheva and Roufa, 1994; Zhao et al., 1994). Mutant human cells having deletions at either near the promoter (Kitsberg et al., 1993) or locus control region (LCR) (Aladjem et al., 1995) of the  $\beta$ -globin locus fail to initiate DNA replication from the origin located within the  $\beta$ -globin locus.

On the contrary, several studies suggested that transcription and DNA replication are antagonistic events. A head-on collision between a replication fork and transcribing RNA polymerase complex arrests or pauses replication in yeast (Brewer et al., 1992; Deshpande and Newlon, 1996), in a similar way to *E. coli* (Liu et al., 1993; Liu and Alberts, 1995). Origin recognition complex

(ORC), the eukaryotic replication initiator, represses transcription of certain genes in yeast (Bell and Stillman, 1992; Micklem et al., 1993; Foss et al., 1993; Bell et al., 1995), although the role of ORC in the repression was recently shown to be separable from its role in replication initiation (Fox et al., 1997).

A similar inverse correlation occurs in *Xenopus* ribosomal RNA genes, in which replication initiation is specifically repressed within transcription units and limited to nontranscribed regions, whereas the replication randomly initiates throughout the transcribed and nontranscribed sequences in early embryos (Hyrien et al., 1995).

Taken together, these observations strongly suggest a tight link between transcription regulation and positioning or firing of replication origins in the chromosomal context. However, how transcription and replication interact with each other, and what biological system(s) the interaction is involved in mammalian cells are still unknown. In this review, we describe our approach to characterize replication origin at the rat aldolase B locus. We also discuss on DNA elements required for replication initiation and on the possible correlation between regulatory systems of replication and transcription.

## **II. The rat aldolase B gene: function, structure and liver-specific expression**

Before focusing on replication origin, we start by briefly describing about the gene coding for aldolase. Aldolase is an enzyme acting on fructose-1,6-bisphosphate metabolism in a processes of glycolysis and gluconeogenesis. The enzyme is a tetrameric protein composed of a combination of three different subunits, A, B and C which are distributed in different tissues and organs in animals (Horecker et al., 1972). Expression of the gene encoding the aldolase B subunit (AldB) is cell type-specific and is under control of cell differentiation; the gene is preferentially expressed in the liver, kidney and jejunal mucosa in adult animal, but the expression is repressed at an early fetal stage and in dedifferentiated hepatocellular carcinomas (Horecker et al., 1972; Numazaki et al., 1984; Tsutsumi et al., 1985; Sato et al., 1987). Studies on the mechanisms operating in such a regulated expression revealed the importance of at least four cis-elements (sites A, B, C, and D) on the proximal 200 bp promoter in the liver-specific transcription, to which a number of regulatory factors interacts cell type-specifically or ubiquitously (**Fig. 1**).

For example, bindings of HNF1 to site A, AIF-B or NF-Y to site B, and C/EBP or AIF-C to site C seem to confer liver-specific transcription (Tsutsumi et al., 1989; Ito, Ki et al., 1990; Raymondjean et al., 1991; Gregori et al., 1993; Tsutsumi et al., 1993; Yabuki et al., 1993; Gregori et al., 1994). Site D acts as a silencer-like element upon transfection, but its role is still unknown (Gregori et

al., 1993). On the other hand, in AldB non-expressing rapidly dividing cells, a different set of factors bind to these sites though their functions are not fully known, e.g., site B and site C bind growth-inducible factors Ryb-a (Ito, Ki et al., 1994) and an alternate type of AIF-C (Yabuki et al., 1993), respectively (discussed later). Thus, various set of regulatory factors interact with the proximal 200 bp promoter, determining cell type-specificity and the level of transcription.

## **III. An initiation region of DNA replication encompasses promoter of the AldB gene in AldB non-expressing rat hepatoma cells**

Regulated state of eukaryotic genes is achieved through the assembly of specialized, heritable chromatin structure with confined domains on chromosomes, which might have causal relationship with locus control region (LCR), insulator, nuclear matrix-association, and positioning of replication origins etc. It is also suggested that regulatory pathways that govern transcription and initiation of DNA replication affect each other or cross-talk (DePamphilis, 1993b; Hamlin et al., 1995; Stillman, 1996). Based on these considerations, we initially thought that transcriptional repression of the AldB gene in rapidly dividing cells, such as fetal liver and hepatoma cells, might somehow relate to initiation of replication. For a step toward understanding the functional and positional relationship between transcription and replication, we tried to identify the replication initiation region nearest to the AldB gene.

To locate initiation region of replication, newly replicated DNA chains were labeled with bromodeoxyuridine (BrdU) using synchronously cultured rat hepatoma dRLh84 cells. BrdU-substituted DNA has higher density as compared to unsubstituted parent DNA and can be separated by ultracentrifugation through CsCl density-gradient. Cells arrested at G1/S boundary by double-thymidine-block were released from the arrest to enter S phase, and cultured in a fresh medium containing BrdU. After various time period, BrdU-labeled DNA was prepared by CsCl isopycnic centrifugation after digestion with an appropriate restriction enzyme, and hybridized with probes corresponding to various regions in and around the AldB gene region. These experiments showed that (i) replication of the AldB gene region starts at mid-S phase, and (ii) the initiation region locates near or within the AldB gene region. Further analysis of the newly replicated short DNA fragments prepared by alkaline sucrose density-gradient centrifugation revealed that (iii) the initiation region expands about 1.5 Kb or less, which encompasses transcription promoter of the AldB gene (**Fig. 1**) (Zhao et al., 1994).

#### **IV. Specific sequence is required for replication of plasmids carrying the AldB origin fragments**

In vivo analyses of newly replicated DNA identified an origin region of DNA replication which encompassed promoter of the AldB gene. Since several mammalian

origins have been reported to possess activities to replicate autonomously (for example, Ariga et al., 1987; McWhinney and Leffak, 1990; Wu et al., 1993), we tried to examine whether the AldB origin fragment promotes replication in a plasmid form upon transfection into mammalian cells. For this purpose, large DNA fragments ranging from 4 Kb to 6.3 Kb derived from the AldB origin

**Fig. 1.** Two origin regions A1 and A2 in the vicinity of the AldB locus in rat. Vertical lines represent EcoRI sites and the lengths of the EcoRI fragments are shown in Kb below the map. Lower panel shows structure off the promoter within the replication origin (A1) region. Transcription factors that interact with the promoter are also shown. Bent arrow indicates position and direction of the AldB gene transcription.

**Fig. 2.** Replication of a plasmid carrying origin fragment (A1) in transfected cells. Plasmids carrying in vivo origin fragments from - 5.7 Kb to + 0.625 Kb (pBOR6.3) or from - 0.675 to + 0.263 Kb (pBOR0.94) were co-transfected with pUC19 or pBOR6.3, respectively, into Cos-1 cells. After the transfected cells were cultured for 72 hr in the presence of BrdU, low-molecular-weight DNA was extracted, digested with EcoRI, and fractionated by CsCl isopycnic ultracentrifugation. DNA in each fraction was separated on an 1%

agarose gel, transferred onto a nylon membrane, and hybridized with a random-primed,  $^{32}\text{P}$ -labeled pUC19 DNA fragment as a probe. LL (light-light) DNA, HL (heavy-light) DNA, and HH (heavy-heavy) DNA indicate unsubstituted, hybrid, and fully substituted DNAs, respectively (see text).

**Fig. 3.** The 200 bp AldB gene promoter is essential for initiation of replication in transfected cells. Various deletion constructs shown in the left panel were transfected together with pBOR6.3 as an internal control and processed as in **Fig. 2**. Replication efficiencies were based on the amounts of HH and HL DNAs in total (HH, HL, and LL) DNAs, and values were expressed relative to the activity of pBOR6.3.

region were inserted into plasmid pUC19, and assayed for autonomous replication in Cos-1 cells based on semiconservative BrdU-substitution of replicating DNA chain. As briefly mentioned above, incorporation of BrdU into replicating DNA increases density of the DNA chain (designated as H chain) and causes it to band at a density higher than that of unsubstituted DNA chain (L chain) in CsCl density-gradient. The transfected cells were grown in the presence of BrdU, then low-molecular-weight DNAs were extracted by the procedure described by Hirt (Hirt, 1967). Newly-replicated, BrdU-labeled DNA was fractionated by CsCl isopycnic ultracentrifugation after digestion with a restriction enzyme, and then subjected to Southern blot hybridization. **Figure 2** shows a typical example of such a replication assay, using a plasmid (designated as pBOR6.3) bearing a 6.3 Kb fragment extending from - 5.7 Kb to +625 bp in bacterial plasmid vector pUC19. In this case, double-stranded DNA with

both strands being replaced by BrdU (HH DNA) appeared in addition to HL and LL DNAs. This means, considering from the semiconservative replication, that at least two rounds of replication had occurred. A negative control plasmid pUC19 co-transfected with pBOR6.3 did not initiate replication, indicating that the observed replication depends on DNA fragment inserted. Plasmid containing the 0.94 Kb fragment from - 675 bp to + 263 bp (pBOR0.94) exhibited similar activity as compared to cotransfected pBOR6.3 (**Fig. 2**, lower panel). Thus, the 0.94 Kb fragment extending from -675 bp to +263 bp seems to have minimum essential components for autonomous replication. Such replication assays were carried out using various deletion constructs and compared their activities to replicate. The results indicated that a 200 bp fragment extending from -200 bp to -1 bp is indispensable to replication initiation, since deletion of the fragment abolished replication activity (**Fig. 3**).

The 200 bp fragment alone could not direct replication. But it restored replication activity when ligated to either upstream (about 500 bp) or downstream fragment (about 300 bp). This observation makes the origin architecture rather complicated. However, since both flanking regions share no similar sequence, it is not conceivable that the same replication elements are present in these two regions. Although entirely unknown at present, one explanation for this may be the presence of different auxiliary elements in each flanking region, both of which have similar activities for replication initiation when they cooperate with the 200 bp sequence (**Fig. 4**).

**Fig. 4.** Three important regions in the predicted origin region. A to D in filled box represent cis-elements for transcription (see **Fig.1**). Ppu and A/T indicate purin-rich element having binding site for a factor Pur  $\alpha$  (PUR consensus sequence) and A/T-rich sequence, respectively. Numbers indicate positions in bp relative to the transcription start site.

In the case of the AldB origin, however, we prefer that specific sequence elements rather than length is required for replication (Zhao et al., 1997). Plasmids used in our replication assays do contain none of mammalian virus DNA sequence, origin of virus DNA, and binding sites for virus T antigen which would activate replication to some extent, so that the observed replication might depend on the AldB origin sequence and is free from initiation machinery for virus DNA replication. Probably, the possibility that quite diverse sets of specific sequence elements can promote firing each of the multiple potential origins on chromosomes might be one of the explanations why no apparently conserved sequence is found in the limited numbers of mammalian origins so far identified (for review, see Stillman, 1996). Indeed, some eukaryotic origins are reported to require sequence-specific interaction of factors to drive initiation (Caddle et al., 1990, Dimitrova et al., 1996). Several transcription factors have been shown to be activators of replication initiation (Li and Botchan, 1993, He et al., 1993, DePamphilis, 1993a). In addition, a 28 kDa factor found in HeLa cell nuclei binds a purin-rich sequence (PUR consensus sequence, discussed later), which is conserved in several origins from yeast, hamster and human to serve initiation of replication as a sequence-

So far, controversial observations concerning plasmid replication in mammalian cells have been reported (reviewed by Coverley and Laskey, 1994). Several of them pointed out, for example, that the length of the DNA template is crucial, rather than sequence; even bacterial DNA in origin-depleted mammalian virus vector replicated in mammalian cells (Krysan and Calos, 1991; Heinzl et al., 1991; Krysan et al., 1993). Further, the fact that no consensus sequences for origins have been found would support the idea.

specific helix-destabilizing factor (Bergemann and Johnson, 1992).

In this view, the AldB origin region has the structural features for potential origins (DePamphilis, 1993b). The 200 bp region at the AldB promoter contains binding sites for multiple transcription factors, the above mentioned PUR consensus sequence (discussed later), and an A/T-rich sequence (Tsutsumi et al., 1989, Zhao et al., 1994).

### **V. Cell cycle-regulated factors bind to the 200 bp region in the AldB origin**

We next intended to know whether the 200 bp region binds factors from hepatoma cells (dRLh 84), in which this region was shown to be centered on an initiation region of chromosomal DNA replication (Zhao et al., 1994)). Within this 200 bp proximal promoter, at least four important cis-elements ( sites A, B, C and D) have been shown to confer tissue- and developmentally specific transcription. Site-A binds both a liver-specific factor HNF-1 and its competitive antagonist HNF-3 (Tsutsumi et al., 1989; Ito, Ki et al., 1990; Raymondjean et al., 1991; Gregori et al., 1993; Gregori et al., 1994), site-B (a CCAAT motif) binds factors AIF-B, NF-Y and a growth-

inducible factor Ryb-a (Tsutsumi et al., 1993; Gregori et al., 1994; Ito, Ki et al., 1994). Site-C binds to C/EBP, DBP and a novel helix-loop-helix protein AIF-C (Yabuki et al., 1993; Gregori et al., 1993; Gregori et al., 1994; and Yabuki et al., manuscript in preparation). Therefore, it is very interesting to examine whether or not binding of the factors to the 200 bp region is under control of cell proliferation and cell cycle progression. For this purpose, growth cycle of rat hepatoma dRLh84 cells was synchronized by double thymidine block (synchrony was monitored by flow cytometry), and their nuclear extracts were prepared every 2 hr after entering S phase. The nuclear extracts were then subjected to gel electrophoretic mobility shift assay using oligonucleotides corresponding to sites A, B, C, and PPU (polypurine sequence containing PUR consensus) as probes. Results showed that in quiescent cells binding activities to these sites were considerably low as compared with those in growing cells. Site A- and site C-binding activities exhibited similar patterns showing veritable cell cycle regulation. Namely, the activities reach a maximal levels at around G1/S boundary, then gradually decrease to the lowest level at late M to early G1 phase. Activity to bind site PPU increases toward S phase. In contrast, site B-binding activity showed only weak change throughout the cell cycle. Thus, in AldB non-expressing hepatoma cells, sites A, C and PPU bind cell cycle-regulated factors whose activities increase prior to S phase while site B binds a factor independently of cell cycle phases. At present, precise characters of these factors are unknown. However, considering from tissue-specific or ubiquitous pattern of expression of the factors that bind to the promoter, site A might bind a factor HNF3 in the hepatoma cells, since another factor HNF1 is not present in these cells (Kuo et al., 1991). Similarly, sites B and C are thought to bind Ryb-a and AIF-C, respectively, because these factors are rather enriched in rapidly growing cells such as fetal liver cells, and induced by growth signals such as partial hepatectomy or serum-stimulation of cultured cells (Yabuki et al., 1993;

Ito et al., 1994; and Yabuki et al., manuscript in preparation).

Since site PPU has PUR consensus sequence, it might bind a factor similar to that binds to PUR consensus, i.e., Pur  $\alpha$  (Bergemann and Johnson, 1992). The factor Pur  $\alpha$  seems to act as a sequence-specific helix-destabilizing factor, and thus implicating in its involvement in initiation of replication. Recently this factor was shown to associate with the retinoblastoma protein Rb, and thus Rb might modulate binding of Pur  $\alpha$  to its recognition site on DNA (Johnson et al., 1995).

These results implied a positive correlation between binding of factors to the 200 bp region and the onset of DNA replication. In dedifferentiated dRLh84 cells, transcription promoter of the AldB gene is completely inactivated, and instead, used as a replication origin (see hypothetical model in **Fig. 5**). One interesting speculation is that preferential binding of the above mentioned factors in AldB non-expressing cells, instead of those usually bind in the liver and activate the AldB gene, leads to repress transcription and consequently promote replication initiation in a cell cycle-dependent manner. Similar observations were reported for *Xenopus* chromosome where embryo-specific origins are inactivated with concomitant activation of nearby transcription units (Hyrien et al., 1995), and for plasmids carrying a human replication origin that inhibition of replication depends on the level of promoter activity (Haase et al., 1994).

## **VI. Chromosomal state of the AldB origin/promoter region**

We have discussed above on identification, sequence requirement, and cell cycle-dependent protein-binding of the replication origin region. In this section, we will focus on the chromosomal state at the AldB origin/promoter region in relation to transcription activity, liver cell proliferation, and development.

**Fig. 5.** Summary of cell cycle- and growth-dependent binding of factors to the AldB gene origin/promoter region. Details are described in the text.

**Fig. 6.** Alteration of chromosomal state at the origin/promoter region during fetal liver development. Horizontal and curved arrows indicate positions and directions of the AldB gene. Vertical arrows marked HS represent DNase I hyper sensitive site. CpG sites which were heavily methylated are shown as Me. Boxes represent promoter regions.

The AldB gene transcription in rat liver is repressed in the fetal stage until around 16th day (day 16) of gestation.

Thereafter, transcription of the gene is drastically activated in the following two to three days (Numazaki et al., 1984).

As expected, the activation during this fetal stage accompanies the alteration of chromosomal state of the AldB gene (see hypothetical model in **Fig. 6**). Comparison of the chromatin structure among those in the livers at the stages when the gene is repressed (day 14), just being activated (day 16), and fully activated (adult) revealed several distinct features for the repressed state (Daimon et al., 1986; Tsutsumi et al., 1987; Ito, Ki et al., 1995; Kikawada et al., unpublished data). Namely, the chromatin had two DNase I-hypersensitive sites II-a and II-b at day 14; the former site disappeared with concomitant activation of the gene. The region around the transcription start site was considerably resistant against DNase I digestion as the fragment derived from the DNase I-hypersensitive sites remained almost intact even after digestion with higher concentration of DNase I; with such a concentration of DNase I the chromatin at later stages was very unstable and was cut into pieces. It was also shown that two CpG sites in HhaI and HpaII sequences near transcription start site are hypermethylated at the repressed stage while those in adult liver are hypo-methylated.

DNase I hypersensitive sites as described above might be a reflection of nuclear matrix association, since the matrix contains topoisomerase II whose cleavage sites in vivo are often found in DNase I hypersensitive regions (Poljak and Käs, 1995). Indeed, several consensus sequences for topoisomerase II cleavage site (Sander and Hsieh, 1985) were found around the DNase I-protected and hypersensitive regions, in addition to clusters of A- or T-rich stretches which are often found in matrix-associated DNA (Gasser and Laemli, 1986) (Fig. 6). With this respect, further analyses using cultured cells encapsulated into agarose beads were carried out to define matrix-associated region. The results suggested that in proliferating, AldB non-expressing cells the DNase I-protected region in the AldB chromatin, i.e., promoter/origin region, is within the matrix-associated region (MAR) (reviewed by Boulikas, 1995). It is not surprising to consider that the origin region is attached to nuclear matrix where replication initiates in proliferating cells, since MAR has been thought to be, for example, an origin of replication (Amati and Gasser, 1988), a boundary of DNA loops demarcating a regulatory domain including a set of transcription units (Laemli et al., 1992), and transcription enhancer elements (Gasser and Laemli, 1986).

Based on these observations, it would be considered that the alteration of chromatin state of the AldB gene promoter/origin region during fetal liver development reflects a change in the organization of functional domains in chromosome. If so, positioning of replication origins and chromosomal domain, for example, might differ between AldB expressing and non-expressing cell nuclei. In this regard, we recently found another origin region at more than 40 Kb downstream of the promoter/origin region in rat hepatoma cells. Both origins are fired in AldB non-expressing hepatoma cells, whereas the downstream origin

was not used in AldB expressing differentiated hepatoma cells, suggesting different organization of chromosomal domains (Miyagi et al., unpublished observation).

## VII. Perspectives

Here, we have described that the transcription promoter of the aldolase B gene is centered on an initiation region of DNA replication in rat hepatoma cells in vivo. Within the origin region, the 200 bp promoter fragment extending from -200 bp to -1bp was indispensable to autonomous replication when assayed by transfection of plasmids bearing various origin fragments. Since the 200 bp fragment alone did not confer replication, the fragment is thought to cooperate with the flanking sequences to play an important role in initiation of replication. The 200 bp promoter consists of multiple cis-elements for liver-specific transcription. In rat hepatoma cells, in which the AldB gene is completely inactivated, protein factors bound to the cis-elements in a cell cycle- or growth-regulated manner, suggesting the involvement of the 200 bp region in regulation of replication initiation. Thus, the promoter of the AldB gene has dual roles in regulation of both liver-specific transcription and initiation of replication. The results, however, do not confine actual "start point" of replication. The 200 bp region, for example, could not necessarily be a start point but rather be an auxiliary element. Further, whether the start point resides at a single location or distributes throughout the origin/promoter region is unknown. These points remain to be elucidated.

Firing of replication origin either at or neighboring the AldB locus, or both, might influence the transcriptional state of the gene, since these biological reactions in a single specialized DNA domain are together regulated according to a DNA loop model, a chromosome model of topologically independent DNA loop domains which are separated by periodic association with nuclear matrix (Laemli et al., 1992). Concerning to this, the observation that the origin/promoter region is attached onto nuclear matrix in vitro and in vivo implies the importance of the region in assembly of functional domains in a chromosome, since MAR has been postulated to act as a replication origin and, in addition, as an insulator-like element in that it reduces position effect in transgenic mice (for example, McKnight et al., 1992). Thus, we think that repression and activation of the AldB gene might reflect positioning of replication origins and alteration of domain structure in chromosomes. In fact, as mentioned earlier, usage of the two origins in the vicinity of the AldB locus differs between the AldB expressing and non-expressing cells. It would be quite important to know how transcription and replication reflect each other in the chromosomal context, since the mechanism that govern such a causal relationship might be involved in cell differentiation and development. For this purpose, the AldB gene promoter/replication origin would be one of the suitable targets.

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