Asymmetric bidirectional replication at the human *DBF4* origin

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Abstract

Faithful replication of the entire genome once per cell cycle is essential for maintaining genetic integrity, and the origin of DNA replication is key in this regulation. Unlike that in unicellular organisms, the replication initiation mechanism in mammalian cells is not well understood. We have identified a strong origin of replication at the *DBF4* promoter locus, which contains two initiation zones, two origin recognition complex (ORC) binding sites and two DNase I-hypersensitive regions within ~1.5 kb. Notably, similar to the *Escherichia coli oriC*, replication at the *DBF4* locus starts from initiation zone I, which contains an ORC-binding site, and progresses in the direction of transcription toward initiation zone II, located ~0.4 kb downstream. Replication on the opposite strand from zone II, which contains another ORC-binding site, may be activated or facilitated by replication from zone I. We term this new mammalian replication mode ‘asymmetric bidirectional replication’.

DNA replication in prokaryotes and lower eukaryotes usually initiates from a discrete *cis*-acting DNA element termed the origin of DNA replication (*ori*)^{1,2}. There are lines of evidence that replication in the mammalian cells also starts from a defined location called an origin of bidirectional replication (OBR)^{2–5}. However, there is equally compelling evidence that replication initiates from a broad initiation zone^{6–9}. It is now generally accepted that several potential *ori* sites exist within a small or large initiation zone, although certain sites within the zone may be preferentially used^{10–12}. However, there is no experimental data that can reconcile the OBR and diffused/delocalized *ori* models. Even in the case of known mammalian OBRs, the exact mode of replication is still to be demonstrated, since the resolution of all known OBRs is several hundreds to thousands of nucleotides range, except the one in the human lamin-B2 (*LMNB2*) locus⁵.

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AUTHOR CONTRIBUTIONS

J.R. planned, developed protocols, carried out experiments, analyzed data and drafted the manuscript; H.L. conceived and guided the overall research project, analyzed and interpreted data, and wrote the final version of the manuscript.

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Although ori sites of mammalian cells apparently do not contain a common motif or consensus sequence, several pronounced features are often found at replication initiation loci¹³. These include asymmetric AT stretches¹⁴, CpG islands¹⁵ and transcriptional control elements^{16–19}. As the first two features are also frequently found at transcription promoter regions, there is a close interplay between regulation of transcription and initiation of DNA replication.

We have been studying DNA replication using the *DBF4* locus as a model. Dbf4 is the regulatory partner of the Cdc7 serine/threonine protein kinase, which is essential for the activation of individual ori sites throughout S phase in eukaryotic cells^{20–22}. After the assembly of pre-replication complexes onto an ori, Cdc7-Dbf4 kinase activates initiation of DNA replication in conjunction with S phase-specific cyclin-dependent kinases²¹. Cdc7-Dbf4 activity is tightly regulated as part of the many mechanisms that cells have in place to ensure that DNA replication occurs at the right time and only once per cell cycle²³. As the abundance of the Cdc7 catalytic subunit remains largely constant throughout the cell cycle, the regulation of Cdc7-Dbf4 activity is thought to occur primarily through modulation of the Dbf4 regulatory subunit^{21,23}.

To understand how Dbf4 is regulated in mammalian cells, we and others previously identified and characterized the *DBF4* promoter^{24,25}. We found that the 237-bp DNA segment from –211 to –447 (that is, upstream of the translation start-codon numbered as +1) has nearly full promoter activity. The *DBF4* promoter contains putative binding sites for several transcription factors, including a MluI cell-cycle box (MCB) and binding sites for Sp1 (four sites) and TFIIB. Among these elements, the MCB (–250 to –245) is essential for the basal promoter activity, and the Sp1 site at –361 to –353 (Sp1 box 3) is required for efficient promoter activity²⁴. In addition, a putative enhancer is found within the 865-bp DNA segment from –1,628 to –994, in which there is a 184-bp DNA segment (–1,186 to –1,003) containing several A-T tracts²⁴ (Supplementary Fig. 1 online). The *DBF4* locus also contains a CpG island (Fig. 1a). Of note, the putative mitochondrial carrier protein gene (*MCFP*), which is located ~20 kb upstream of the *DBF4* gene, may also use the *DBF4* promoter for its transcription²⁵. The activity of the *DBF4* promoter increases substantially at the G1/S transition²⁶. These features of the *DBF4* promoter led us to postulate that it may contain an ori with high activity in early S phase.

Here, we report the mapping of an ori and replication initiation points (RIPs) in the *DBF4* promoter region. Replication at this locus initially starts from initiation zone I and then proceeds in the sense direction (that is, the direction of *DBF4* transcription) toward initiation zone II. The replication of antisense strand from initiation zone II may occur only when the replication on the sense strand has reached or passed through this initiation zone. This mode of replication in human cells is notably similar to that at the bacterial *oriC* locus, raising the possibility that asymmetric bidirectional replication may be common at ori sites of many organisms.

RESULTS

The *DBF4* promoter region contains a strong ori

To determine whether the *DBF4* promoter contains an ori, we carried out a nascent strand abundance assay²⁷. To avoid DNA damage during nascent strand preparation, we directly loaded HeLa cells into a gel, where they were lysed. DNA was then separated by gel electrophoresis under denaturing conditions, followed by isolation of short nascent strands (1–2 kb) from the gel as described previously²⁸. The analysis of nascent-strand abundance was carried out by quantitative PCR (Q-PCR) using primer pairs corresponding to a large part of the *DBF4* locus, including the promoter, putative enhancer and part of the coding region (Fig. 1a and Supplementary Table 1 online). DNA segments corresponding to primers Prom7B (–675 to –625) and Prom8 (–151 to –100) were 8–10 times more abundant than adjacent regions (Fig. 1b). In contrast, no substantial enrichment was found for any sequence using mitotic cells or sonicated total genomic DNA (Fig. 1b). These data suggest that an ori exists within the 574-bp DNA segment from –675 to –100, which includes the principal transcription initiation site (–235) (ref. 24). We obtained similar results with HEK293 and HEK293T cells (data not shown). We found that initiation activities at the *DBF4* locus in HeLa cells were at least 2–3 times greater than those of the *MYC* (*c-myc*) (not shown) or lamin-B2 ori (Fig. 1b). In addition, data from synchronized HeLa cells showed that the *DBF4* ori fired extensively for a relatively short period in early S phase (7 h after mitosis or 1 h after G1/S arrest) (Fig. 2), providing an excellent model for studying mammalian DNA replication.

The *DBF4* ori contains two separate initiation zones

Precise replication initiation points (RIPs) at the human lamin-B2 locus have been determined using the technically challenging ligation-mediated (LM) PCR method⁵. To our knowledge, however, no other mammalian RIP has thus far been mapped by LM-PCR since that study was published in the year 2000. This is probably due, at least in part, to the difficulties of employing LM-PCR for RIP mapping.

A different research group successfully used a common one-way PCR-based primer extension method to map RIPs at the *II/9A* amplified ori of *Sciara coprophila*^{29,30}. We therefore examined one-way PCR-based primer extension on the well characterized lamin-B2 ori in an attempt to develop technically a less challenging mammalian RIP mapping method. Using emetine-treated synchronized HeLa cells, we were able to detect a leading strand RIP of the ‘bottom’ strand at the expected position⁵ (Supplementary Fig. 2 online). Emetine was used to detect synthesis of the leading strand only, as it inhibits Okazaki fragment synthesis without affecting leading strand progression³¹.

As the *DBF4* locus showed strong initiation activities (Fig. 1b), we determined leading strand RIPs in asynchronous HeLa cells using one-way PCR-based primer extension. Data from 1- to 2-kb nascent strand templates with primer A (pA, corresponding to –106 to –125; Supplementary Table 2 online) suggested that approximate replication start-sites on the sense strand were –390, –400, –485, –510 and –565 (Fig. 3a). As we detected no primer extension product with pB (corresponding to –796 to –815), replication initiation may not

occur upstream of -815 on the sense strand (Fig. 3a). Data obtained with pC (corresponding to -130 to -111) suggested that RIPs on the antisense strand were located at or around +130, +310, +330, +370 and +460 (Fig. 3a). Primer extension with pD (corresponding to -675 to -657) amplified a single ~800-nt strand, suggesting that replication initiated around +130 on the antisense strand (Fig. 3b,c). Other RIPs detected using pC were not detected with pD, presumably because the primer extension by one-way PCR was not optimal to amplify a long template. However, the main result of the primer extension with pD was that no RIP smaller than 800 nt was detected, even when smaller products were actively sought (Fig. 3b, right panel). Together, our data suggest that the initiation at the *DBF4* locus does not follow the conventional OBR model, since no initiation was observed on the opposite strand from RIPs detected with pA (Fig. 3c).

Next, we determined RIPs of synchronized HeLa cells in the presence (or absence, in some cases) of emetine (Fig. 4a). When pA was used for primer extension with a sample taken at 1 h after G1/S in the absence of emetine, a replication transitional point was detected at -235. (This position is calculated by adding -106 (pA position) and -129 (the approximate size of the band determined by the gel electrophoresis)). In addition to the band corresponding to -235, many primer extension products were observed in this non-emetine-treated sample (Fig. 4a). This was expected, as Okazaki fragments would ligate to the leading strand DNA as replication continued. However, it seemed that some of the bands might not have been generated by ligation of Okazaki fragments because certain high-molecular-mass bands were unusually strong. Consistent with this interpretation, the sample treated with emetine also showed several primer-extension products. Together, our data suggested that leading strand synthesis on the sense strand started from several sites, including -235, -255, -260 and -390 (Fig. 4a, panel pA). Combining the RIP data from synchronous and asynchronous cells, we conclude that there are multiple initiation sites within the DNA segment amplified with pA. It should be noted that the products generated by one-way PCR-based primer extension were not the results of nonspecific amplification of randomly cleaved genomic DNA, as no product was generated from emetine-treated mitotic cells (Fig. 4a). Before the RIP assays, we also tested primers using cloned template DNA to ensure that polymerization was not prematurely terminated under our experimental conditions (data not shown).

Consistent with data from asynchronous cells, no RIP was detected with pB or pY (corresponding to +421 to +440) in synchronized cells, confirming that initiation does not occur upstream of -815 or downstream of -235 on the sense strand (Fig. 4a). Similarly, replication initiation on the antisense strand starts around +130, +265 and +290, but not upstream of +130 (Fig. 4a, panel pC).

To pinpoint the leading strand start-points, we determined precise RIPs using sequencing-gel electrophoresis. For this experiment, HeLa cells synchronized at the G1/S interface were released for 1 h into complete medium containing emetine. One-way PCR-based primer extension with 0.5–1.0-kb long leading-strand templates showed 49 RIPs on the sense strand, all of which clustered within a 420-bp segment from -264 to -683 (Fig. 4b,c; Supplementary Table 3 online). Among these RIPs, initiation of DNA replication with a C residue was dominant (20/49, 41%), followed by T (11/49, 22%), G (10/49, 20%) and A

(8/49, 16%). In a similar experiment, we found that replication initiation on the antisense strand started at positions +311, +312 and +320 (Fig. 4b,c).

Together, our data suggest that there are two initiation zones at the *DBF4* ori locus. The 449-nt-long initiation zone I spans from -235 to -683, within which at least 49 RIPs were detected. The 331-nt-long initiation zone II, on the antisense strand, stretches from +130 to +460 and contains only a few RIPs. Replication initiation was not detected within the 365-bp space between these two initiation zones (that is, from -235 to +130), suggesting that replication initiation seldom occurs within this DNA segment.

Asymmetric initiation of DNA replication at the *DBF4* locus

Because replication from initiation zones I and II occurs in opposite directions and progresses through the overlapping 'no-initiation' zone (Fig. 4c), replication initiation on the sense and antisense strands may not occur simultaneously. To gain insight into potential asymmetric replication, we carried out RIP assays with small (0.5–1.25 kb) and large (1.25–2.0 kb) nascent-strand templates isolated from cells synchronized at early S phase. RIPs were detected from the smaller templates amplified with either pA or pC (Fig. 5a). Primer extension with pA also showed several RIPs from the larger templates. In contrast, no RIPs were detected with pC from the larger templates, raising the possibility that DNA synthesis on the sense strand may initiate earlier than that on the antisense strand (Fig. 5a).

To further examine the possibility of asymmetric replication, we conducted primer extension using short single-stranded DNA templates (0.5–1.0 kb) isolated from HeLa cells that had been released from G1/S arrest for 1 or 2 h in the presence of low concentrations of aphidicolin (and emetine). Because aphidicolin can effectively slow down DNA chain elongation (but not initiation) at a low concentration, it has been used to determine the initial direction of DNA synthesis at the SV40 ori locus³². At 0.15 $\mu\text{g ml}^{-1}$ aphidicolin, we detected several RIPs using both pA and pC at 1 h after G1/S (Fig. 5b, left panel). However, we detected no RIPs with either primer by 2 h after G1/S, suggesting that all of the nascent DNAs initiated on both strands had progressed beyond detection (that is, > 1.0 kb) by 2 h after G1/S. This conclusion is consistent with the data shown in Figure 2d. When replication forks were further slowed down by increasing the concentration of aphidicolin to 0.5 $\mu\text{g ml}^{-1}$, RIPs on the sense strands were detected at 1 and 2 h after G1/S with pA (Fig. 5b, right panel). In contrast, no appreciable replication activity was detected with pC at either 1 or 2 h after G1/S under the same conditions (Fig. 5b, right panel). These data suggest that replication from zone I on the sense strand occurs before initiation from zone II on the antisense strand.

ORC binds replication initiation zones I and II

Binding of the origin recognition complex (ORC) to an ori is a prerequisite for 'licensing' the initiation of DNA replication^{2,33} by which the positions of replication initiation may be determined²⁹. To begin examining whether *DBF4* ori is bound by ORC, we carried out chromatin immunoprecipitation (ChIP) with antibodies to ORC4 (Supplementary Methods online). DNA segments corresponding to Prom7C2 and HS(A) primer sets (Supplementary Table 1) were most abundantly bound to ORC (Fig. 6a). The abundance of flanking regions

gradually decreased in both directions from the two peaks. These data therefore suggest that there are two ORC-binding sites: one at –608 to –529 (corresponding to Prom7C2) and the other at +131 to +182 (corresponding to HS(A)). The ORC-binding sites and other features of the *DBF4* ori/promoter are summarized in Supplementary Figure 1.

Two pronounced DNase I-hypersensitive regions in *DBF4* ori

To gain insight into the chromatin structure at the *DBF4* locus, we carried out DNase I sensitivity assays (Fig. 6b). The DNA segment corresponding to primer AT-2, but not two flanking regions (Prom6 and Prom7B), were DNase I hypersensitive, suggesting that the DNA segment spanning –1,081 to –947 (AT-2) has an ‘open’ chromatin structure. The segment corresponding to Prom4 (–467 to –398) within initiation zone I was also DNase I hypersensitive. The open chromatin structure at this locus extended to Prom8 (and to HS(A) to some extent); however, the openness gradually decreased as the distance from the Prom4 site increased further downstream (Fig. 6b,c). The downstream boundary of this DNase I-hypersensitive region approximately coincides with that of initiation zone II (Fig. 6c).

DISCUSSION

Identification of an ori at the *DBF4* promoter region

The existence of a strong ori at the *DBF4* promoter region was initially determined by a nascent-strand abundance assay and then confirmed by RIP mapping. Although the same locus was identified by these two independent methods, the boundary of the ori identified by the former is largely confined within initiation zone I (see below for discussion).

We found that one-way PCR-based primer extension³⁰ could be very sensitive and powerful in determining a RIP when combined with the following sample preparation methods: (i) lysis of mammalian cells directly in the gel to avoid DNA damage; (ii) isolation of nascent strands directly from gel after DNA separation by gel electrophoresis under denaturing conditions, which gives a high yield of short single-stranded DNAs; and (iii) treatment of cells with emetine so that only leading strands are generated and isolated. Because PCR is routinely used in many laboratories, this PCR-based RIP mapping approach may greatly facilitate the study of mammalian DNA replication.

Identification of replication initiation zones I and II

One of the most notable features of the *DBF4* ori was that it contained two initiation zones, separated by ~400 bp no-initiation zone (Fig. 4). Many replication initiation sites were clustered within the 449-nt initiation zone I (–683 to –235), whereas only few initiation sites occurred within the 331-nt initiation zone II (+130 to +460). Although we cannot completely rule out the possibility that some of the leading-strand RIPs detected in the emetine-treated cells were generated by ligation of Okazaki fragments to the leading strand DNA, most of the RIPs, if not all, are likely to be genuine replication initiation sites. This interpretation is supported by the fact that certain bands in the non-emetine-treated sample showed very strong intensities. If these bands were generated by ligation of Okazaki fragments to the leading strand DNA, the signal intensities of the bands should have gradually decreased as the lengths of leading strand increased. However, we did not see this pattern.

The upstream boundary of initiation zone I approximately coincided with the ORC binding site (−608 to −529), and the downstream boundary overlapped the putative TFIIB- and MCB-binding sites (−267 to −261 and −250 to −245, respectively)²⁴. The second ORC-binding site (+132 to +182) also roughly coincided with the upstream boundary of initiation zone II. Thus, ORC binding may determine the boundary of an initiation zone, rather than determining the precise positions of individual RIPS. We found that although specific nucleotides were generally used, slightly different initiation sites were observed between synchronized and asynchronous cell populations (summarized in Fig. 4c) as well as among different experiments (data not shown). However, the overall boundaries of the initiation zones never varied, suggesting that that chromatin context but not nucleotide sequence is essential for the activation of initiation in mammalian cells.

In early S phase, numerous RIPS were detected in the shorter (0.5–1.25 kb) nascent strands within initiation zone I, but only a few RIPS were found in the larger (1.25–2.0 kb) nascent strands (Fig. 5a). A similar trend was also found in the cells treated with low concentrations of aphidicolin to slow down replication fork movement (Fig. 5b). Together, these data raise the possibility that the half-life of the short leading strands in initiation zone I is unusually long, perhaps because of a transient pause of the replication fork (see below for discussion). This interpretation is supported by the fact that the *DBF4* ori identified by the nascent strand abundance assay was largely confined within initiation zone I.

A new model: asymmetric bidirectional replication at the *DBF4* locus

Initiation of DNA replication starts from initiation zone I more frequently and earlier than from zone II. This makes sense because it would be easier for the DNA to replicate, at least initially, in the same direction as that of vigorously transcribing genes such as *DBF4*. In addition, the chromatin structure at or around initiation zone I was more open than that around zone II, which could facilitate the assembly of the replication protein complexes at this region. Consistent with this expectation, initiation zone I contained a strong ORC-binding site. It seems possible that the open chromatin structure facilitates the binding of ORC to the upstream boundary of initiation zone I, allowing the chromatin to become competent for replication initiation (Fig. 7a). The chromatin in this region may become further competent for DNA replication by the binding of transcription factors at the downstream boundary of zone I (that is, binding of ORC at one end of initiation zone I and binding of transcription factors at the other end).

The Sp1 transcription element has been shown to positively affect DNA replication initiation in virus and yeast^{34–36}. Our data Figure 4c is in line with these previous reports, potentially expanding the positive role of Sp1 on DNA replication to mammalian cells. In contrast to Sp1, MCB (and possibly TFIIB) may negatively affect replication initiation (and, thus, define the downstream boundary of initiation zone I). It may be possible that the MCB transcription factor forces the replication fork movement to stall transiently while MCB ‘negotiates’ with the replication machinery before letting it pass through the downstream boundary. The initiation of DNA replication on the antisense strand could be suppressed by transcription factors bound to the chromatin and/or high transcriptional activities in the opposite direction in early S phase. It is possible that DNA replication from initiation zone I

can temporarily disrupt the transcriptional machinery bound at the promoter and, thus, provide a short ‘window of opportunity’ for antisense replication. It is therefore likely that the initiation on the antisense strand can occur only when the replication fork on the sense strand has reached or passed through initiation zone II (Fig. 7b–d). We term this newly identified mode of mammalian DNA replication asymmetric bidirectional replication (ABR).

OBR, diffused/delocalized initiation, and ori of ABR

An important question raised by our work is whether ABR is a general phenomenon or an exception. We cannot definitely answer this question until RIPs of many more mammalian ori sites have been characterized in details. It is, however, possible that many mammalian ori sites known at present (including some OBRs) may actually replicate asymmetrically. The maximum resolution of replicon-mapping techniques available at present, except RIP mapping, is 0.5 to 1.0 kb. At this low resolution, one cannot definitely conclude how many initiation sites or zones an ori contains, or whether replication initiation occurs asymmetrically. When the ori at the *DBF4* locus was identified by a nascent strand abundance assay alone, we did not suspect asymmetric replication to occur at this locus. We realized the *DBF4* ori operated through ABR only when we systematically mapped the RIPs.

Only a single ORC-binding site is found at the lamin-B2 locus, where a single OBR operates. In contrast, the *DBF4* locus contains two initiation zones, each of which has ORC-binding site. Because ORC may determine the position of replication initiation sites²⁹ or initiation zones (this report), the number and position(s) of ORCs at the ori locus could be the determinant of the OBR or ABR replication mode. Overall, ABR replication at the *DBF4* does not extend to the lamin-B2 ori, instead showing a clearly different paradigm.

Although we have unequivocally shown in this paper that the *DBF4* locus contains two initiation zones, it could have been mistaken as a single (or two) OBR(s) under different assay conditions (for example, using only nascent-strand abundance assay, or using different synchronization time points and/or sizes of nascent strand DNA templates, and so forth). It is also possible that low-resolution replicon-mapping methods could identify the *DBF4* locus being a ‘diffused or delocalized’ initiation zone because replication bubbles could be detected within the entire 1.5 kb DNA segment from –1,000 to +500. Thus, the ABR model, at least in part, can explain and reconcile the OBR and the diffused or delocalized replication initiation models.

ABR in other organisms

Notably, the ABR replication pattern found at the *DBF4* locus is very similar to that at the *E. coli oriC* locus³⁷. *In vivo* studies showed that leading strand replication in *E. coli* initiates at multiple sites within and around *oriC*. In addition, approximately 250 bp between the two initiation zones at the *oriC* locus is largely devoid of initiation³⁷. Also similarly to the *DBF4* ori, the two initiation zones at the *oriC* locus seem to initiate in a temporally separable manner³⁷. Although replication at *oriC* shows a more classical OBR pattern in the plasmid context³⁸, the mode of *oriC* replication in the chromosomal context is highly similar to that

of *DBF4*. (This may suggest that the chromosomal context is important in determining initiation sites even in bacteria.) Furthermore, a similar mode of multiple initiations and asymmetric replication has also been found at *ori* sites of mouse mitochondria and the amplified II/9A of *S. coprophila*^{29,39}. With these data taken together, ABR may be quite common in organisms, including mammals. In this context, we note that it has recently been hypothesized⁷ that *oriβ* and *β'* at the *Dhfr* locus in the Chinese hamster ovary cell lines may regulate replication similarly to *oriC*. In light of our findings, their hypothesis makes sense, especially if several sets of origins of ABR exist in the *Dhfr* locus.

METHODS

Nascent DNA isolation

We carried out separation and isolation of short single stranded nascent DNA by alkaline gel electrophoresis as described previously²⁸ with some modifications. Briefly, approximately 10^7 cells were collected by trypsinization, washed once with $1 \times$ PBS and then resuspended in 260 μ l of $1 \times$ PBS containing 10% (v/v) glycerol. To prevent DNA damage during sample preparation, the cell suspension (85 μ l) was directly loaded into a well of the 1.25% agarose gel containing 50 mM NaOH and 1 mM EDTA. The cells were then immediately lysed by adding 25 μ l of $5 \times$ SDS lysis buffer (50 mM Tris HCl buffer, 5 mM EDTA, 0.5% (w/v) SDS, pH 8.0). After incubation for 10 min at room temperature, EDTA was added to a concentration of 20 mM and then NaOH to 50 mM. Electrophoresis was carried out for >8 h at 15 V at room temperature in freshly made alkaline running buffer (50 mM NaOH, 1 mM EDTA). After neutralization with $1 \times$ TAE (40 mM Tris, pH 8.0, 20 mM acetic acid, 1 mM EDTA) for 45 min, the lane containing DNA size markers was separated from the rest of the gel and stained with $1 \times$ TAE buffer containing $0.6 \mu\text{g ml}^{-1}$ ethidium bromide for 15 min. The size of the nascent DNA in the unstained gel was determined by comparing it with the ethidium bromide-stained DNA size marker. Subsequently, nascent DNA fragments were extracted from the gel using a QIAGEN gel-extraction kit.

Nascent strand abundance assay by Q-PCR

Q-PCR was carried out using a 7900HT ABI Prism (Applied Biosystems) in 25 μ l Q-PCR solution containing 12.5 μ l of $2 \times$ SYBR Green Master Mix (Applied Biosystems) and 300 nM of each primer (Integrated DNA Technologies). Standard curves were generated for each primer pair using serial dilutions of total genomic DNA. An equal volume of each nascent strand preparation was used for amplification. After initial denaturation for 10 min at 95 °C, amplification was carried out for 40 cycles as follows: 95 °C for 30 s, 57 °C for 30 s and then 72 °C for 30 s. Amplification of a single product at a correct DNA size by a given primer pair was verified by agarose gel electrophoresis. Relative abundance of nascent strands was estimated by calculating the ratio between the amount of each DNA segment (as estimated by the standard curves) and the amount of the DNA amplified by the Prom3 set (which consistently showed the lowest amplification).

RIP mapping

To analyze RIPs of leading strand synthesis, 2 μ M emetine (Sigma) was added to the cell culture media for 1 h before DNA isolation. Where indicated, aphidicolin (Sigma) was also

added. An equal volume of each DNA preparation (or an equal amount measured by Q-PCR when comparing different DNA preparations) was used as template in the primer extension reactions with 5'-digoxigenin-labeled primers. One-way PCR reactions were carried out in a final volume of 30 μ l containing 1 $\times$ PCR buffer (ID Labs Biotechnology), 200 μ M dNTPs, 3 mM MgSO₄, 400 nM digoxigenin-labeled oligonucleotides (Integrated DNA Technologies), 1 M betaine (Sigma) and 1 unit of IDPol DNA polymerase (ID Labs Biotechnology). For the RIP mapping of the lamin-B2 ori, reactions were carried out in the absence of betaine. PCR conditions were as follows: initial incubation for 5 min at 95 °C, 30 cycles of amplification (1 min at 94 °C, 1 min at the appropriate annealing temperature as shown in Supplementary Table 2, and 1.5 min at 72 °C) and final extension for 7 min at 72 °C. As a negative control, a sample without template DNA was included in every experiment. After amplification, 20 μ l of 'sequencing' loading buffer (98% (v/v) formamide, 10 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue) were added to each sample. Samples were then heated for 4 min at 90 °C, chilled quickly on ice and separated by PAGE in a gel containing 5% acrylamide and 7.7 M urea in 44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.4. After transfer of DNA onto a Hybond N⁺ membrane (Amersham) for 1 h at 400 mA using a semi-dry transfer apparatus, the DNA was fixed with UV for 6 min. Detection of digoxigenin-labeled PCR products with an antibody to digoxigenin (Roche) was performed according to the manufacturer's instructions.

DNase I hypersensitivity assay

The isolation of nuclei and subsequent limited digestion with DNase I were carried out as described previously⁴⁰. Briefly, asynchronously growing HeLa cells (~10⁷) were trypsinized, resuspended in 1 ml RSB buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂) containing 0.2% (v/v) NP-40, and incubated for 5 min on ice. Nuclei were isolated by centrifugation for 5 min at 500g. The nuclear pellet was resuspended in 600 μ l of RSB buffer, and then divided into six 100- μ l aliquots. To each aliquot, an appropriate amount of DNase I (Invitrogen) was added, and the aliquot was incubated for 5 min at 37 °C. The digestion reaction was stopped by adding an equal volume of Stop solution (1% (w/v) SDS, 600 mM NaCl, 10 mM EDTA, 20 mM Tris HCl, pH 8.0). Proteins in each sample were digested overnight with Proteinase K (Sigma), followed by phenol-chloroform extraction and ethanol precipitation. Finally, purified DNA was resuspended in 10 mM Tris HCl, pH 8.0, 1 mM EDTA, and quantified by spectrophotometry (Beckman DU-530).

Q-PCR was performed using 8 ng of DNA template for each sample. Standard curves were generated using serial dilutions of independently isolated, undigested total genomic DNA. Reaction mixture (25 μ l) contained 12.5 μ l of 2 $\times$ SYBR Green Master Mix and 300 nM of each primer. After amplification, the amount of target DNA segment remaining in the digested samples was determined using the standard curve. The results were compared to the amount detected in the control sample that was not treated with DNase I.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The *DBF4* promoter locus contains a strong ori. (a) Numbers are relative positions to the A (+1) of the translational start-codon. The numbering in brackets is according to the CTB-60N22 BAC clone. The *DBF4* major transcription initiation site²⁴ is shown as a vertically elongated triangle. The putative transcription initiation site for MCFP is shown as a broken line. Primers used to quantify nascent strands are shown as converging arrows (Supplementary Table 1). Gray and white in the *DBF4* promoter are the core and auxiliary elements, respectively. (b) *DBF4* ori is localized within the DNA segments corresponding to Prom7B and Prom8. The relative abundance of nascent DNA was quantified by Q-PCR and was normalized to the amount of nascent DNA amplified with Prom3. Lamin-B2 ori was included as positive control. Gray, white and black are nascent DNAs from asynchronous cells, nascent DNAs from mitotic cells and sonicated total DNA, respectively. Error bars, s.e.m. of at least three independent experiments. * $P < 0.01$.

Figure 2.

Replication initiation at the *DBF4* locus is largely confined to around 7 h after mitosis or 1 h after G1/S. **(a)** Flow cytometric profile of DNA content in postmitotic HeLa cells. M, cells arrested at mitosis by nocodazole. As, asynchronous cells; N, haploid DNA content. **(b)** Short nascent DNA corresponding to Prom8 is the most abundant at 7 h after mitosis. Nascent strands (1–2 kb) were isolated at the indicated time points after HeLa cells were released from nocodazole-based mitotic arrest. The relative abundance of DNA corresponding to each primer set was measured by Q-PCR. **(c)** Flow cytometric profile of post-G1/S HeLa cells. 0 h, cells arrested at the G1/S boundary by double-thymidine treatment. **(d)** Replication at the *DBF4* ori (corresponding to Prom8) is most active 1 h after G1/S.

Figure 3.

Determining RIPs at the *DBF4* locus in asynchronous HeLa cells. **(a)** Primer extension with the indicated digoxigenin-labeled primers (pA, pB and pC) using nascent DNA (1–2 kb) isolated from asynchronous HeLa cells that had been incubated for 1 h with emetine (As). L, digoxigenin-labeled molecular weight ladder; N, no template; arrow, the same RIP identified by both pC and pD (+130 position); MW, molecular weight. **(b)** Replication initiation on the antisense strand in asynchronous HeLa cells occurs far downstream from initiation zone I. Left panel, nascent strand templates (1–2 kb) were subjected to PCR-based primer extension with pD. The resultant products were separated by gel electrophoresis to resolve high-MW fragments. Lanes: 1, MW marker; 2, 5'-digoxigenin-labeled PCR product (734 nt); 3, no sample; 4, sham reaction control including primer pD but no template; 5, primer extension on nascent DNA templates (annealing temperature, 64 °C). Arrow, DNA amplified by primer extension with pD. The gel at right in **b** was designed to resolve smaller DNA fragments. Lanes: 1, MW ladder; 2, no sample; 3, sham reaction control; and 4, the same as in lane 5 of the left panel. **(c)** Summary of data in panels **a** and **b**. Note that pA and pB primers are on the sense strand and pC and pD are on the antisense strand.

Figure 4.

DBF4 ori contains two initiation zones within ~1.1 kb DNA segment. (a) RIPs of leading strands determined by primer extension using nascent DNA templates (1–2 kb) isolated from HeLa cells. Cells arrested at the G1/S interface were released for 1 h into medium containing emetine (lane 2). Primers used (pB, pA, pY, pD and pC; Supplementary Table 2) were 5'-end labeled with digoxigenin. Control PCR reactions were performed in the absence of template (lane 1) or using DNA isolated from emetine-treated, mitotic cells (lane 3). Two samples were not treated with emetine (lane 4 in panels pA and pD). MW, molecular weight; L, digoxigenin-labeled MW ladder; M, digoxigenin-labeled 738-nt PCR product as an extra MW marker. (b) RIP assays performed with 0.5–1.0 kb nascent DNA (+) from cells at 1 h in S phase (see above) or in the absence of template (–). Arrows, some of RIPs and their first nucleotides. (c) Summary of initiation sites. Vertical dashed and solid lines, leading-strand DNA initiation sites as determined from data in a and b, respectively. The taller vertical lines are RIPs with stronger signals. Gray arrows, replication directions from initiation zones I (sense strand) and II (antisense strand). Black dots, the RIPs of asynchronous HeLa cells (from Fig. 3a).

Figure 5.

Replication initiation occurs from replication zone I before zone II. **(a)** At 1 h after G1/S, much more abundant RIPs were detected from replication zone I than zone II. RIP analysis was carried out using short (0.5–1.25 kb) or long (1.25–2.0 kb) nascent DNA isolated from emetine-treated HeLa cells 1 h after G1/S. MW, molecular weight; L, MW ladder; pA and pC, the digoxigenin-labeled primers used for primer extension. **(b)** RIP assays carried out with 0.5–1.0-kb nascent DNA isolated from HeLa cells treated as in **a**, except that aphidicolin was added at the indicated concentrations. NTC, no template control.

Figure 6.

Mapping of the ORC-binding and DNase I-hypersensitive sites. **(a)** ORC binds preferentially to the segments corresponding to Prom7C2 and HS(A). The binding of ORC to DNA in asynchronous HeLa cells was determined by ChIP with antibodies to ORC4. The abundance of DNA segments in ChIP was determined by Q-PCR. The resultant values were standardized by subtracting the amount recovered with preimmune IgG. Relative abundance was calculated as percentage of input DNA and then normalized to the values obtained for Prom3 primer. * $P < 0.05$. **(b)** To map DNase I-hypersensitive sites, HeLa nuclei were treated with different concentrations of DNase I. Eight nanograms of DNA from the nuclei were quantified by Q-PCR with primers pairs indicated. Standard curves were generated for each primer pair using total, undigested genomic DNA to determine the amount of DNase I-resistant DNA. A nuclease-sensitive region of the *MYC* (*c-Myc*) promoter was a positive control⁴¹. Error bars, s.e.m. of two or more experiments. **(c)** Summary of the ORC-binding and DNase I-hypersensitive regions.

Figure 7.

An ABR initiation model. **(a)** The numbers and map shown are as described in the Figure 1 legend. The elongated circles and the associated double line are putative nucleosome locations and duplex DNA, respectively. The Sp1 binding site (−353/−361) and the MCB (−250/−245) were described previously²⁴. ORC denotes ORC-binding sites. **(b–d)** The detailed ABR initiation mechanism is described in the text. Long and short arrows represent nascent leading and lagging strands, respectively.