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Discrete Functional Elements Required for Initiation Activity of the Chinese Hamster Dihydrofolate Reductase Origin Beta at Ectopic Chromosomal Sites

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Abstract

The Chinese hamster dihydrofolate reductase (DHFR) DNA replication initiation region, the 5.8 kb ori-beta, can function as a DNA replicator at random ectopic chromosomal sites in hamster cells. We report a detailed genetic analysis of the DiNucleotide Repeat (DNR) element, one of several sequence elements necessary for ectopic ori-beta activity. Deletions within ori-beta identified a 132 bp core region within the DNR element, consisting mainly of dinucleotide repeats, and a downstream region that are required for ori-beta initiation activity at non-specific ectopic sites in hamster cells. Replacement of the DNR element with *Xenopus* or mouse transcriptional elements from rDNA genes restored full levels of initiation activity, but replacement with a nucleosome positioning element or a viral intron sequence did not. The requirement for the DNR element and three other ori-beta sequence elements was conserved when ori-beta activity was tested at either random sites or at a single specific ectopic chromosomal site in human cells. These results confirm the importance of specific *cis*-acting elements in directing the initiation of DNA replication in mammalian cells, and provide new evidence that transcriptional elements can functionally substitute for one of these elements in ori-beta.

Keywords

DNA replication; origin; DHFR; dinucleotide repeat; transcription factor; ori-beta

INTRODUCTION

More than 40 years ago Jacob *et al.* [1] proposed a DNA replicon model which led to the discovery of replicators from bacteria to mammals [2,3]. A replicator is a *cis*-acting genetic element that directs replication initiation to occur at a specific location recognized by a *trans*-acting initiator. Budding yeast *Saccharomyces cerevisiae* use DNA replicators that contain a short consensus sequence that interacts with the origin recognition complex (ORC) [4]. Genetic, biochemical, and physical mapping of origins of DNA replication on mammalian

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chromosomes suggests the existence of replicators that may specify DNA replication initiation sites in mammalian cells [5–10]. Moreover, ORC and its role in the initiation of DNA replication are conserved from yeast to mammals, indicating that mammalian replicators might share some features with those of budding yeast [11–19]. However, unlike replicators in budding yeast, the *cis*-acting sequence elements that contribute to initiation activity of mammalian replicators have no consensus sequence except for a small AT-rich sequence bias [2,3,20].

To identify a chromosomal replicator in mammalian cells, an origin DNA fragment is placed at an ectopic chromosomal site and assayed for its capacity to direct initiation of replication at the ectopic site. To achieve this, two general strategies have been used. Using a non-specific integration system, the 5.8 kb Chinese hamster DHFR origin beta (ori-beta) [8,9] and the 1.2 kb human LaminB2 origin [21] showed replication initiation activity at multiple chromosomal sites in mammalian cells. These functioned as active replicators in pooled stably transfected cells or individual cell clones, although DNA replication activity varied at different chromosomal sites. The second strategy employed FLP- or Cre-mediated specific recombination integration systems to introduce the 2.4 kb human c-myc origin [7,10] and the 2.6 and 3.2 kb human beta-globin origins [6,20,22] into unique ectopic chromosomal sites in a human cell line and a mouse cell line, respectively. The c-myc and beta globin origins serve as replicators at their specific ectopic chromosomal sites. Whether the two different strategies are equally effective in identifying mammalian replicators has so far not been validated by directly comparing them with the same mammalian origin.

One of the obstacles to understanding mammalian origins is the lack of identifiable sequence homology between different origins, but even in budding yeast replicators, some elements are not conserved, e.g. the B2 element in ARS1 [23] and the binding site for the Abf1 transcription factor in the ARS1 replicator [24–28]. Interestingly, an Sp1 binding site can functionally replace the Abf1 site in ARS1 [29], suggesting that yeast replicators lacking an Abf1 site contain other elements that may serve the same functional role in directing initiation of replication. Moreover, these results suggest a hypothesis that may explain the DNA sequence diversity among mammalian replicators and that can be experimentally addressed.

The mammalian replicators characterized so far are composed of multiple sequence elements, of which several are required for initiation activity of the replicator. Five sequence elements identified within the 5.8 kb DHFR ori-beta fragment are each necessary, but not individually sufficient, for full initiation activity of ori-beta at ectopic locations in Chinese hamster cells [8,9]. These include a 4 bp GGCC within a GGGCCC palindrome within the peak of initiation activity, an AT-rich element (AT), a CA+GA dinucleotide repeat element (DNR), a region of bent DNA, and a binding site for the 60 kDa Replication Initiation Protein (RIP60) (Figure 1). A sixth element (not shown) was dispensable for activity [8]. Since each mutation caused a loss of ori-beta activity while the other elements remained intact, each mutated or deleted element represents a separate component critical for ectopic ori-beta initiation activity. Importantly, the hamster ori-beta AT element could be functionally replaced by a non-homologous sequence from the human laminB2 origin [9], consistent with the hypothesis that dissimilar DNA sequences from different mammalian replicators may serve similar functional roles. Further support for the concept that replicator elements that differ in DNA sequence can functionally substitute for each other comes from the ability of a Gal4-binding site to replace a 1.4 kb element in the 2.4 kb ectopic c-myc replicator in HeLa cells expressing Gal4-CREB, but not Gal4 alone [30].

In this report, we have mapped DNA sequences in the DNR element of hamster ori-beta that are crucial for full initiation activity of the ori-beta replicator at random ectopic chromosomal sites in hamster cells. We present evidence that either the *Xenopus* 5S ribosomal RNA gene or

an element from the murine 28S ribosomal RNA regulatory region can replace the function of the DNR element to restore ori-beta initiation activity. Lastly, we demonstrate the requirement for DNR and the other ori-beta elements for replicator activity at ectopic chromosomal sites in human cells, using both the specific and non-specific integration strategies.

MATERIALS AND METHODS

Plasmid construction

The plasmid pMCD containing DHFR ori-beta, a 5.8-kb BamHI-KpnI fragment in pUC19, was the kind gift of N. Heinz [31]. Mutant constructs pMCDΔDNR, pMCDΔAT, pRIP DSx, and pBENDx were described previously [8,9]. Full-length DHFR ori-beta and its mutant derivatives were subcloned into the plasmid pFRT.Myc [10] to replace the BamHI-NotI fragment including the 2.4 kb c-myc replicator, and named pFRT.ori-beta, pFRT.DNR, pFRT.AT, pFRT.RIP, and pFRT.BENT, individually. The DNR reversed mutant (pMCD-DNRrev) was generated by digesting pMCD with NheI and partially with XbaI to release the DNR fragment, then ligating its compatible cohesive ends back into the vacant DNR location in pMCD and screening for integrations in the opposite orientation. The resulting DNRrev mutation was subcloned from pMCD into the pFRT vector to make pFRT.DNRrev. The XK deletion mutant was made by digesting pMCD fully with KpnI and partially with XbaI, to delete a 1.3 kb fragment from nucleotide 4454-5781 (relative to the BamHI site) to make the plasmid pMCDΔXK.

Construction of the following DNR mutants used the same strategy: pMCD-0.5DNR1, pMCD-0.5DNR2, pMCD-DNRsper, pMCD-SB2r, pMCD-5SRNA, pMCD-5SNPEf, and pMCD-5SNPEr. The SB2 insert [32,33] came from the plasmid pUC-SB2+ and was the kind gift of F. Grummt. The 5S RNA insert [34] came from the plasmid pXP10 and was the kind gift of J.J. Hayes. Each mutant construct used a derivative of pMCD (pMCD-X) in which the TCTAGA XbaI site at the downstream border of DNR was changed to a CCCGGG XmaI site for easier cloning. For each construct, the DNR element was removed by NheI/XmaI digestion, and the vacant gap was filled by ligation of a PCR-amplified fragment flanked with NheI- and XmaI-compatible cohesive ends. PCR primers used to amplify the replacement fragments are provided in Table 1. All changes to the normal ori-beta sequence were verified by sequencing. The DNRsper mutation was subcloned from pMCD into the pFRT vector to make pFRT.DNRsper.

Cell culture and stable transfection

Hela S3 (ATCC CCL-2.2) and DR12 [35] cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37°C with 10% CO₂. Hela 406 cells, a kind gift from M. Leffak, were previously derived from Hela cells by integration of an FLP recombinase target site (FRT) [7] and maintained in Dulbecco's modified Eagle medium with 10% FBS and 50 μg of gentamicin/ml at 37°C with 5% CO₂.

Four μg of pMCD plasmid DNA or a mutant derivative plasmid mixed in a 3:1 molar ratio with PvuI-linearized pSV2neo DNA, were electroporated into either 5 x 10⁶ Hela or DR12 cells using a Bio-Rad Gene Pulser at 360 V and 650 μF. After 3–4 weeks of growth under G418 (0.5 mg/ml) selection, ~100–200 G418 resistant clones per transfection were pooled for further analysis.

Hela 406 cell transfections were performed using Lipofectamine 2000 (Gibco-BRL) according to the manufacturer's instructions. For each transfection in a 24-well plate the total amount of DNA was 1 μg; the molar ratio of the donor plasmid to the cotransfected, FLP recombinase-expressing plasmid, pOG44, was 1:8. At 24- to 48-hrs post-transfection, selection for G418

resistance (0.5 ng of active component per ml of culture medium) was initiated and continued for 15 days. After single colonies formed, 20 μ M ganciclovir was supplied for 2 to 3 days. Colonies resistant to hygromycin, G418, and ganciclovir were used for further analysis.

Diagnostic PCR screening for site-specific integration in the Hela genome

After using the site-specific FRT-mediated integration strategy, single-clone colonies resistant to hygromycin, G418, and ganciclovir were screened for site-specific integration by diagnostic PCR as previously described [10]. Briefly, genomic DNA isolated from resistant colonies was amplified by PCR with the diagnostic primer sets 1+2 or 1+3 (Figure 1C). A PCR amplicon that spans the FRT acceptor site using the 1+2 primer set will be detected when no DNA is integrated. Upon integration of DNA at the FRT site the amplicon size becomes too long to amplify under the PCR conditions used. A PCR amplification product from primers 1+3 can be detected only when the Neo^R gene has integrated next to the Hyg^R gene at the FRT site.

Southern blots

For the non-specific integration system, 10 or 40 μ g genomic DNA from uncloned pools of pMCD-transfected cells was digested with BamHI/KpnI and electrophoresed through a 1% agarose gel. The DNA was blotted on a Highbond N+ (Amersham #RPN203B) and crosslinked using a Stratagene UV Stratalinker 1800. The blot was prehybridized in 15 mL Church buffer (0.5 M Na₂HPO₄, 1 mM EDTA, 7% SDS, 1% BSA) at 65° C for several hours. The ectopically integrated ori-beta fragment was detected using two ori-beta probes, probe 1 and probe 2. Probe 1 is a 486 bp BamHI/StuI fragment at the 5' end of 5.8 kb ori-beta, and Probe 2 is a 484 bp NsiI/KpnI fragment at the 3' end of 5.8 kb ori-beta. The GNAI3 probe is a 505 bp PCR fragment from the GNAI3 locus in Chinese hamster cells (nt 4623 to 5128, Genbank Accession # X79282) amplified by the following primers: 5' ATGCTAATTGTAGTAGTGATCC and 5' CCTCAAAGGCACTGCTCC. The GNAI3 probe hybridizes to a 3.5 kb BamHI/KpnI fragment. Fifty ng of each probe fragment was radiolabeled with 50 μ Ci [α -³²P]dCTP (3000Ci/mmol, PerkinElmer LAS # BLU513H250UC) using the High Prime labeling reagent (Roche # 11585592001). The radiolabeled probe (~50 ng at 1–2 x10⁶ cpm/ng) was heat denatured in the presence of 750 μ g salmon sperm DNA, added to the prehybridization solution, and hybridized overnight at 65° C. The blot was washed: twice with 2X SSC (300 mM NaCl, 30 mM NaCitrate) and 0.1% SDS for 15 min each at 25° C, once with 1X SSC/0.1% SDS for 15 min at 25° C, and four times with 0.1X SSC/0.1% SDS for 5 min each at 65° C. The washed blot was exposed to a phosphor imager screen to detect the radioactive signal.

For the specific integration system, Southern blot analysis was performed using standard methods with 10 μ g of genomic DNA and hybridizing with either the 756 bp EcoRI/XbaI fragment of the Hyg gene from plasmid pFRT.Hyg.TK or the 405 bp NcoI/SmaI fragment of the Neo gene from pFRT.myc, as previously described [10].

Nascent DNA isolation and PCR-based nascent DNA strand abundance assay

For the non-specific integration system in DR12 or Hela cells, ~0.4–2 kb nascent DNA isolation using neutral sucrose gradient size fractionation was carried out as previously described [8,9]. Target sequence quantitation in nascent DNA-enriched fractions was done using competitive PCR for Hela samples as described [8,9] or by Real-time PCR for DR12 samples. To test the initiation activity of origin constructs at the specific integration site at Hela chromosome 18, 1–2 kb nascent DNA was isolated by alkaline agarose gel size chromatography as previously described [10], and target sequence quantitation of a nascent-enriched DNA fraction was done using Real-time PCR. Primer sequences are provided in Table 2.

The LightCycler FastStart DNA master SYBR Green I kit (Roche # 12239264001) was used for Real-time PCR quantitation following the manufacturer's instructions. All reactions were

carried out on a Roche diagnostic real-time PCR LightCycler. The total volume of each PCR reaction was 10 μ l with 3.5 mM Mg⁺⁺, 500 nM of each primer (Integrated DNA Technologies, Inc), and 3 μ l of sample DNA. Reactions were started with 10 min at 95° C, 3 cycles using the highest annealing temperature, and 3 cycles using the intermediate annealing temperature, followed by 39 cycles using the lowest annealing temperature. Fluorescence measurements were taken only during the last 39 cycles. During the final 3-sec step of each cycle when fluorescence was measured, the temperature was elevated to minimize any signals emitted by primer dimers and non-specific PCR products with melting temperatures below that of the target. Calibration curves were generated using serial dilutions of a known concentration of pre-sheared genomic DNA from the 406/pFRT.ori-beta cell line when using the specific integration system, or *ScaI*-linearized pMCD when using the non-specific integration system.

RESULTS

Characterization of the DNR element

Deletion of DNR from the 5.8 kb DHFR ori-beta at random ectopic chromosomal sites in DR12 hamster cells resulted in a 9-fold decrease in initiation activity [8]. To assess whether all or part of the DNR sequence was specifically required for full ori-beta activity or whether the DNR element simply maintained critical spacing between neighboring elements, we created further deletion and substitution mutants of DNR. Each construct was stably integrated at random locations in DR12 cells, and total genomic DNA from pooled transfected cells was isolated. Integration of the intact 5.8 kb BamHI/KpnI ori-beta fragment into the DR12 hamster genome was monitored by Southern blot using a fragment at the GNAI3 locus as a loading control. In two independent transfections, full-length ori-beta was detected at about 1/3 the level of the endogenous ori-beta in CHO-K1 cells (Figure 2A). Nascent DNA of ~0.4–2 kb was obtained by size fractionation of denatured genomic DNA from asynchronously growing, pooled transfected DR12 cells. The abundance of target sequence in the nascent DNA fractions was determined by real-time PCR using primer sets at the ori-beta initiation site (pp2) and at an outlying site ~3 kb away (pp3) (Table 2, Figure 2C).

Using 5.8 kb wild type (WT) ori-beta (pMCD) as a positive control and the DNR deletion mutant as a negative control, we measured the initiation activity of ori-beta mutant constructs (Figure 2D). Replacement of the 235 bp DNR element with a 235 bp fragment from an SV40 intron (DNRspcr) was not able to rescue initiation activity. Reversing the original orientation of DNR (DNRrev) reduced the initiation activity at ori-beta, but it remained easily detectable (Figure 2D). These results indicate that some sequence-specific feature of DNR is critical for its role in ori-beta initiation activity at random ectopic locations in DR12 cells.

The 235 bp NheI-XbaI DNR sequence is composed of GCTA (CG)₅(CA)₁₉(GA)₂₀(G)₁₀(CAGA)₄GGGAGAGAGGCAGAGAGGG(GA)₂₈ followed by 44 bp of sequence without any repeats. *In vitro* studies of the DNR sequence showed that the (CG)₅(CA)₁₉ sequence can adopt a left-handed Z-DNA conformation, and the two (GA)_n sequences can adopt triplex DNA structures [36]. To further define functional regions within the DNR element, we deleted either the first half (0.5DNR2) or the second half (0.5DNR1) of the DNR sequence and tested the initiation activity of each of these ori-beta constructs in DR12 (Figure 2D, rows v-vi). The 0.5DNR1 deletion, which removed the (GA)₂₈ and downstream sequences, had only a minor reduction of initiation activity as compared to the WT ori-beta construct. In contrast, the 0.5DNR2 deletion, which removed the upstream half of DNR up to the CAGA repeats, reduced initiation activity to 25% of WT ori-beta. These results indicate that most of the functionality of DNR resides within the first half of its sequence, but that there is a smaller contribution from the second half.

To test if additional functional elements exist downstream of DNR, the entire ~1.3 kb XbaI-KpnI (XK) downstream region was deleted. This XK mutant showed initiation activity levels equivalent to the DNR deletion, indicating little or no initiation at ori-beta (Figure 2D, row vii). This result suggests that at least one additional ori-beta functional element is located downstream of DNR.

Two dissimilar transcriptional elements can independently replace the function of the DNR element

The importance of DNR in the initiation activity of ectopic ori-beta might also be explained by the activity of DNR as a replication fork barrier (RFB), since early studies of the DNR region revealed that it impedes the progression of replication forks emanating from an SV40 origin in an orientation-dependent manner [37]. To test this possibility, the DNR element was replaced with a known RFB from the murine 28S rDNA locus [32,33], such that it would impede replication forks moving toward ori-beta (Figure 3B, row ii). This 161 bp element contains a binding site for the Transcription Termination Factor 1 (TTF-1), which binds to the second Sal box (SB2) motif at the rDNA locus [38]. In addition to its role in termination of transcription and replication elongation, TTF-1 also acts to remodel chromatin and activate transcription [39,40]. When the initiation activity of the SB2 replacement mutant was evaluated at ectopic sites in DR12 cells, ori-beta initiation activity was above that of WT ectopic ori-beta (Figure 3C, row ii), indicating that the SB2 element is able to functionally replace DNR.

The non-conserved distal Abf1-binding element in yeast ARS1 is known to limit the ability of nucleosomes to mask the ORC-binding site, thereby inhibiting ARS1 activity [41]. Given the distal location of DNR in ori-beta, we wondered whether DNR might also position nucleosomes to facilitate protein binding to elements necessary for ori-beta activity, such as AT. To address this possibility, the DNR sequence was replaced with the 282 bp *Xenopus* 5S RNA gene, which contains a well-characterized nucleosome-positioning element (NPE) [34]. The orientation of the 5S RNA gene replacement sets the direction of transcription away from ori-beta. The 5S RNA gene (5Sf) replacement of DNR displayed initiation activity above that of WT ori-beta (Figure 3C, row iii), indicating that the 5S gene was able to functionally replace DNR.

We next sought to gain more insight into the mechanism of 5S RNA gene function as a DNR replacement. The 5S gene could stimulate ori-beta by positioning nucleosomes, by recruiting transcription factors via its Transcription Factor IIIA (TFIIIA) binding site, or by providing a specific DNA structural conformation [42]. To distinguish among these possibilities, new ori-beta constructs were designed in which DNR was replaced by a 5S RNA gene lacking the TFIIIA binding site and downstream sequences, but with the NPE left intact. This partial 5S RNA gene was inserted in both orientations in place of DNR, and the initiation activity of the resulting ori-beta mutant constructs at random ectopic sites in DR12 cells was determined. Both constructs lacking the TFIIIA binding site had sharply reduced initiation activity relative to the complete 5S RNA gene replacement, with minor initiation activity detected in the forward, but not reverse, orientation (Figure 3C, rows iv-v). These results suggest that the functional replacement of DNR by the 5S RNA gene is dependent on its TFIIIA binding site, and not solely on its ability to position nucleosomes.

The DNR element is required for ori-beta activity at a specific integration site in human cells

Our characterization of DNR used stably integrated ori-beta constructs at random chromosomal locations, but we cannot rule out the possibility that the requirement for various sequence elements in ori-beta activity depends on the random integration strategy that we used or on the hamster cell environment. In an attempt to confirm the results of Figures 2 and 3 with ori-beta integrated at a specific chromosomal site in a different mammalian species, we generated a panel of ectopic ori-beta constructs at the same specific chromosomal site in the HeLa acceptor

cell line 406. Ectopic wild type and mutant ori-beta cell lines with the DNR deletion, DNR orientation reversal (DNRrev), and DNR substitution with an identically-sized DNA fragment spacer from an SV40 intron (DNRsper) (Figure 4) were integrated at the same chromosomal site in the Hela acceptor cell line 406, so the resulting lines were completely isogenic except for the mutation introduced in each construct. After G418 and ganciclovir screening, the resistant clones were verified by diagnostic PCR analysis (Figure 4A). The acceptor cell line 406 showed the specific band using diagnostic primers 1 and 2. Conversely, when the FRT site was occupied with a large ori-beta DNA fragment mediated by FLP recombinase, primers 1 and 2 did not amplify the corresponding band. But primers 1 and 3, located within the Neomycin resistance gene, amplified the expected band in all positive ori-beta clones. Southern blot analysis of EcoRI-digested genomic DNA from of these clones used two probes, Hyg and Neo, to verify the integration location and integrant copy number (Figure 4B). Hybridization of the Hyg probe to a 5.6 kb EcoRI fragment confirmed that integration occurred at the intended chromosomal location (Figure 4B, left). Hybridization of the Neo probe solely to the 5.6 kb band in the ori-beta lines indicated that specific integration in the acceptor FRT site had taken place (Figure 4B, right).

Once each ori-beta construct was successfully integrated at the specific site in Hela 406, the initiation activity of ori-beta in each cell line was measured by real-time quantitative PCR using three primer sets, pp2b, ppHyg, and ppGlobin (Figure 1, Table 2). Primer set ppGlobin, located at the human beta-globin locus at chromosome 11, was used as an internal reference to compare different cell lines and nascent DNA preparations. In the WT ori-beta line, the abundance of target sequences in nascent DNA detected with pp2b was ~15-fold higher than that detected with ppGlobin and ~10-fold higher than that with the outlying ppHyg (Figure 4D), indicating that ori-beta is active at the specific ectopic site in Hela 406 cells. Deletion of the DNR element or substitution with a spacer abolished ori-beta initiation activity, confirming that the specific sequence of DNR is critical to its role in regulating DNA replication initiation (Figure 4D). Reversing the original orientation of DNR did not diminish replication initiation at ori-beta (Figure 4D).

Other ori-beta elements contribute to initiation activity in human cells

Full initiation activity of the ectopic ori-beta replicator in DR12 hamster cells was shown to require specific DNA sequences in an AT rich element (AT), in the downstream RIP60 binding site (RIP), and in the bent DNA element (BENT) [9]. To assess the importance of these elements for the initiation activity of ori-beta at random ectopic chromosomal sites in human cells, Hela cells were stably transfected with each of the mutant constructs (Figure 5). Fractions enriched in 0.4–2 kb nascent DNA were prepared from uncloned pools of these transfected cells and the abundance of four ori-beta target sequences in each fraction was determined by competitive PCR. Initiation activity was calculated by normalizing the abundance of each target to that of an outlying target 3 kb away from the initiation site (pp3). In Hela cells, initiation activity of wild-type ectopic ori-beta at pp2 was about 40-fold greater than that pp3 (Figure 5C). In contrast, the initiation activity of ectopic ori-beta lacking the AT element was greatly reduced. Moreover, the mutations of downstream RIP60 binding site (RIPx) and the bent DNA structure (BENDx) decreased the initiation activity at pp2 to a level similar to that at pp3. These results are consistent with those obtained using a similar strategy in DR12 hamster cells [9].

We then examined the activity of three ori-beta mutants integrated at a specific ectopic chromosomal site in Hela 406 cells. Integration of these ori-beta constructs at the specific integration site was confirmed by PCR and Southern blot analysis (Figure 6A, 6B). Compared to the WT ori-beta control, the AT element deletion, RIPx mutation, and BENDx mutation each virtually eliminated ori-beta initiation activity at the pp2b site, with no enrichment seen above that of the ppHyg and ppGlobin sites (Figure 6D). The results confirm that these three

elements are essential for ori-beta activity in human and hamster cells, at either random or specific ectopic chromosomal sites.

DISCUSSION

Ori-beta constructs can be evaluated using different ectopic integration strategies

In this report, we have measured the Chinese hamster DHFR replicator ori-beta DNA initiation activity in HeLa and DR12 cells at specific and at random ectopic chromosomal sites. Consistent with previous results [8,9], our investigation showed that the 5.8 kb DHFR initiation region ori-beta is active at ectopic sites not only in hamster cells, but also in human cells (Figures 2 and 5). Mutations that disrupted ori-beta activity in DR12 or HeLa using the non-specific integration system also disrupted initiation in the specific site in HeLa (compare Figures 2 and 4, 5 and 6). These results provide evidence that the potential pitfalls of non-specific integration, including position effects, do not obscure or falsify the determination of ectopic origin function.

Transcription through the endogenous ori-beta has been shown to inactivate the origin [43, 44]. However, transcription of the neomycin resistance gene upstream of the site-specific integrated origin constructs in the HeLa 406 line should not be a concern, since a strong SV40 transcription terminator is located between the neomycin resistance gene and the integrated origin fragment [7,45]. Therefore, transcription elongation across the ectopic ori-beta would not be expected to suppress its activity, and this is supported by the ori-beta initiation activity seen in the integrated WT ori-beta construct (Figure 4).

The 3' end of the 5.8 kb ori-beta fragment contains at least two sequence elements necessary for ori-beta initiation activity

Deletion of either the 235 bp DNR element or the 1.3 kb XK region downstream of DNR led to a loss of ori-beta initiation activity at non-specific chromosomal sites in DR12 cells (Figure 2D), and the effect of the DNR deletion was recapitulated at a specific locus in HeLa cells (Figure 4D). The observation that elements >3 kb from the ori-beta initiation site are critical for ori-beta activity is perhaps surprising given that a 1.2 kb fragment from the human LaminB2 origin is sufficient for ectopic initiation activity [21]. However, such a distal effect is not unprecedented given that deletion of the distal hamster DHFR promoter affects replication initiation in the entire downstream 55 kb intergenic region [46].

The loss of initiation activity upon deletion of the 5' half of DNR, but not the 3' half (Figure 6D), suggests that the main functional component(s) of DNR resides within the first 132 bp of its 235 bp length. It also suggests that the loss of initiation activity upon deletion of the XK region is not due to the disruption of a necessary element that overlaps the DNR and XK elements, but rather because at least one separate and novel functional element exists within the 1.3 kb XK element that is not found in the DNR element. The failure to functionally replace the 235 bp DNR element with a 235 bp DNA fragment from an SV40 intron (Figure 2D and 4D) indicates that DNR does not serve just to maintain a critical spacing between flanking elements, but that its DNA sequence is important.

Transcription factor binding sites can functionally substitute for DNR

The specific sequence of DNR, in particular the first 132 bp of its 235 bp length, serves some functional role in the initiation of DNA replication at ori-beta from over 3 kb away from the initiation site. What functional role might it serve? This 132 bp sequence is as follows: GCTAG (CG)₄(CA)₁₉(GA)₂₀(G)₉(CAGA)₄GGGAGAGAGGCAGAGA. The first 6 bp are a palindromic NheI site, and the rest of the sequence, except for the final 16 bp, is repetitive. These final 16 bp, however, were not sufficient for full initiation activity of an ori-beta mutant lacking the preceding repetitive sequences (Figure 2D, 0.5DNR2 mutant). GA repeats have

been shown to impede replication forks [37, 47, 48] and affect the assembly of DNA into nucleosomes [49], so both of these potential roles were investigated separately (Figure 3). Importantly, a replication fork barrier (SB2) and an element that contains a nucleosome-positioning element (5S rDNA) were each able to independently replace the function of DNR (Figure 3C).

The results implied that these two elements share some functional characteristic with the DNR element. The DNR element was previously shown to pause replication forks in an *in vitro* SV40 replication assay [37], suggesting that this could be a common feature shared by DNR and SB2. However, no pausing of replication forks has been described at the extensively studied 5S RNA gene, arguing that replication pausing is not the critical feature shared by these three elements. The protein TTF-1, which binds to the SB2 element, is capable of actively repositioning nucleosomes [40], leading us to suspect that SB2 and the 5S rDNA substituted for a function of DNR in positioning nucleosomes. However, since the NPE from the 5S RNA gene could not, without the TFIIB binding site, functionally replace DNR (Figure 3C), we conclude that SB2 and 5S rDNA must have additional or alternative functions needed to substitute for DNR in ori-beta activity.

The general role of transcription factors is to regulate the formation of various functional complexes at specific chromosomal sites assembled from elements recognized by these factors [50]. The functions of transcription factor binding sites may thus resemble those of replicator elements needed to assemble ORC and pre-replication complexes. Comparison of the two transcription elements that substitute for DNR in the ectopic ori-beta replicator may reveal the function of DNR. The SB2 element contains binding sites for TTF-1 and Ku 70/80 heterodimer. These proteins participate in initiation of rDNA transcription [39,51], termination of transcription and replication [52,53], and TTF-1 can also remodel chromatin [39,40]. The 5S gene contains a binding site for TFIIB, which recruits TFIIC and TFIID to form a stable transcription activation complex on the 5S gene [42,54]. Thus both transcription elements able to substitute for DNR share the ability to gain access to a specific site in chromatin and nucleate assembly of a multiprotein complex at that site. This type of transcription element-dependent chromatin remodeling, in the absence of transcription, facilitates assembly and function of the V(D)J recombination complex at specific times in lymphocyte development [55,56]. We speculate that both the 5S gene and the SB2 element may substitute for DNR by creating a suitable chromosomal environment that is important for the essential functions of the flanking AT or XK elements in ori-beta replicator activity. We further speculate that transcriptional activators such as the hamster Pur1 or human homolog MAZ, which bind GA repeats such as those in DNR [57,58], might interact with DNR and mediate this type of chromatin remodeling.

The function, but not necessarily the DNA sequence, of *cis*-acting elements in a mammalian replicator promotes initiation of DNA replication

One of the obstacles to understanding mammalian origins has been the lack of identifiable sequence homology between different origins. As shown here, the repetitive sequences in DNR are critical for initiation activity of ectopic ori-beta (Figure 2 and 4, [8]), but CA and GA repeats are not commonly associated with replication origins. Moreover, we have shown that the DNR element can be functionally substituted by two different unrelated sequences, and not by a third sequence (Figure 2,3). These data provide additional support for the hypothesis that *cis*-acting elements that together comprise a mammalian chromosomal replicator can have different DNA sequences in different replicators, yet fulfill the same function in origin activity. These data also extend our previous finding that the AT-rich element of ori-beta can be functionally replaced by a non-homologous sequence from the LaminB2 origin [9] by showing that mammalian transcriptional control elements can also substitute for a mammalian replicator element. Detailed characterization of additional mammalian replicators, mutant replicators,

and the proteins that interact with them will be needed in the future to uncover the common functions of replicator elements that differ in sequence.

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Figure 1.

The DHFR origin beta at endogenous and ectopic locations. (A) Diagram of the endogenous DHFR ori-beta IR in hamster cell. Preferred start sites of DNA replication (ori- β , ori- β' , and ori- γ) within a 55-kb initiation zone between the genes DHFR and 2BE2121 are indicated. The 5.8 kb DNA fragment containing ori-beta extends from the BamHI (nucleotide position 1) to the KpnI restriction enzyme site. Previously identified [8,9] functional elements of ori-beta are indicated: IR, initiation region; RIP60, 60 kDa replication initiation protein binding sites; BEND, sequence-induced stable bend in the DNA; AT, (AT)_n repeats and AT-rich sequences; DNR, GA+CA dinucleotide repeat element. (B) 5.8 kb ori-beta sequence cloned into pUC19 (pMCD) and integrated at non-specific ectopic locations in DR12 and Hela cells. Locations of pp8, pp2, pp6 and pp3 PCR primer sites are indicated by gray diamonds. (C) 5.8 kb ori-beta sequence integrated at the FRT site in Hela 406 cells [10]. The locations of the FRT sites, hygromycin-neomycin resistance fusion gene (Hyg^R Neo^R) with transcription start site (bent arrow), poly-adenylation and transcription termination site (pA), and thymidine kinase (TK) gene are indicated. The 5.6 kb EcoRI fragment used for Southern blot analysis, and diagnostic PCR primers 1, 2, and 3 for specific site integration are shown. The target sites for ppHyg and pp2b are indicated with gray diamonds.

Figure 2.

Initiation activity of ori-beta constructs at multiple ectopic chromosomal sites in pooled stably transfected DR12 cells. (A) Integration of full-length ori-beta into DR12 cells was assayed by Southern blot analysis of BamHI/KpnI-digested genomic DNA hybridized to ori-beta probes 1 and 2, or probe GNAI3 as a loading control. “DR12” refers to untransfected cells, “ori-beta CHOK1” refers to the endogenous ori-beta, “ori-beta DR12” refers to stably transfected ori-beta sequences in DR12 cells, and “ori-beta plasmid” refers to 30 pg of pMCD. Densitometry quantitation of ectopic ori-beta was first calculated in each lane as [(intensity of 5.8 kb band)/(intensity of 3.5 kb band)], then expressed as a percentage (shown below the blot) of the same densitometry quantitation done in the CHOK1 lane corresponding to the same μ g quantity of DNA. (B) Summary of the methodology used to prepare and analyze nascent DNA samples. (C) Diagram of ori-beta constructs, with primer sets pp2 and pp3 shown (gray diamonds). (C,

rows I, ii, and vii) Cartoon representation of the DHFR ori-beta, with deleted regions shown as dashed lines. (C, rows iii-vi) DNR replacement and partial deletion constructs, in the context of the entire ori-beta fragment, as described in the Materials and Methods. The DNR region and replacement elements are drawn to scale ± 5 bp. All construct diagrams in (C) correspond to the adjacent name and bar graph in (D). In panel D, "initiation activity" is calculated as the abundance of target sequences detected with primer set pp2 in nascent-enriched DNA fractions, divided by those detected with primer set pp3. For comparison, all initiation activities are expressed as a percentage of the initiation activity of pMCD (unmodified 5.8 kb ectopic ori-beta, row i). Each bar is the average initiation activity measured from at least 3 independent transfections and nascent DNA preparations. Brackets indicate SEM. *, significantly lower than the pMCD construct; +, significantly greater than the Δ DNR construct (student's t-test, p value <0.05).

Figure 3.

Loss of initiation activity in DNR-deleted ori-beta can be restored by transcriptional elements. (A) Summary of the methodology used to prepare and analyze nascent DNA samples. (B) Diagram of ori-beta constructs, with primer sets pp2 and pp3 shown (gray diamonds). The DNR region and replacement elements are drawn to scale ± 5 bp. (B, row i) Wild type ectopic ori-beta in DR12 cells. (B, row ii) Arrows above and below the SB2 element indicate the polar impediment of replication forks moving toward ori-beta (X) mediated by TTF-1 at its binding site (thick line). (B, row iii) A gray arrow indicates the transcription direction of 5S RNA gene, with the nucleosome positioning element (NPE, thick line) and TFIIIA binding site (black box) highlighted. (B, row iv-v) Partial 5S RNA gene replacements containing the NPE (black bar) without the TFIIIA binding site, in either orientation. Dashed lines indicate deleted regions. All construct diagrams in (B) correspond to the adjacent name and bar graph in (C). In panel C, “initiation activity” and notations are as described in the legend of Figure 2.

Figure 4.

Initiation activity of mutant DNR ori-beta constructs at the specific chromosomal site in Hela cells. (A) Following transfection with ori-beta constructs and drug selection, genomic DNA from clonal cell lines was tested for site-specific integration by PCR. A PCR product using primers 1+2 indicates no integration at the FRT site, but a product using primers 1+3 indicates integration in the correct orientation. (B) Southern blots EcoRI-digested genomic DNA from clonal lines was hybridized to either the Hyg probe or Neo probe as described in the Materials and Methods. The Hyg probe hybridizes to a 2.3 kb fragment in the acceptor cell line and to a 5.6 kb fragment when ori-beta is integrated. The Neo probe hybridizes to a 5.6 kb fragment only in the integrated ori-beta. (C) Summary of the methodology used to prepare and analyze

nascent DNA samples as previously described [10]. (D) Analysis of the initiation activity of ori-beta constructs at the specific integration site in Hela. 406, Hela acceptor cell line; WT, full-length ori-beta fragment; Δ DNR, DNR deletion construct; DNRrev, construct in which the DNR orientation was reversed; DNRsPCR, replacement of DNR with a 235 bp SV40 intron fragment. "Initiation activity" is defined as the abundance of target sequences in nascent DNA-enriched fractions detected at primer sets pp2b (black bars) or ppHyg (white bars), divided by that at primer set ppGlobin. Brackets indicate SEM for WT.

Figure 5.

Initiation activity of ori-beta constructs at multiple ectopic chromosomal sites in pooled stably transfected HeLa cells. (A) Diagram of WT ori-beta and mutated residues. The sequences necessary for the bent DNA region (underlined) and downstream RIP60 binding site (bold) are shown. Nucleotide substitutions for the BENDx and RIP60 mutants are in parentheses and brackets, respectively. (B) Summary of the methodology used to prepare and analyze nascent DNA samples as previously described for hamster cells [9]. (C) "Initiation activity" was calculated by dividing the quantity of target sequences in nascent-enriched DNA fractions detected at primer sets pp8, pp2, or pp6 by that detected at primer set pp3. Primer set locations are given in Figure 1B. Construction of these ori-beta plasmids is described in [8,9]. Bars are

the average quantitation of target sequences in at least 2 independent transfections and nascent DNA isolations. Brackets indicate SEM.

Figure 6.

Initiation activity of ori-beta constructs at the specific chromosomal site in HeLa cells. (A) PCR-based analysis of clonal cell lines containing ori-beta constructs, as described in the Materials and Methods section and Figure 4A. (B) Southern blot analysis of EcoRI-digested genomic DNA from clonal cell lines, as described in the Materials and Methods section and Figure 4B. (C) Summary of the methodology used to prepare and analyze nascent DNA samples as previously described [10]. (D) Analysis of the initiation activity of ori-beta constructs at the specific integration site in HeLa. 406, HeLa acceptor cell line; WT, full-length ori-beta fragment. Mutant constructs are described in the Materials and Methods and Figure 5. "Initiation activity" is defined as the abundance of target sequences in nascent-enriched DNA fractions detected at

primer set pp2b (black bars) or ppHyg (white bars), divided by that at primer set ppGlobin. Brackets indicate SEM for WT.

Table 1
PCR primer sequences for the amplification of DNR replacement fragments

Replacement	nt. ^a	Sequence (5' to 3') ^b	Template	Insert (bp)
DNRsper	1202 1429	AGTCTAGACAGCTTTTTCCTTTGTGGTGT CATCCCGGGACTGGTGGGAATGCCTTTAATG	pSV2-neo ^c	235
0.5DNR1	3558 4351	AAGGACCTCAGCCTCTGAAAC ATGCCCGGGTCTCTGCCTCTCTCCCTCTG	pMCD ^d	132
0.5DNR2	4332 5075	CTAGCTAGCACAGAGGGAGAGAGGCAGAG ACCCTGTTCTCTGCTAAGCAG	pMCD ^d	126
5S RNA gene	-79 +212	ATATGCTAGCAATTCGAGCTCGCCCCGG CGAGGTCGACTCTAGAGGA	pXP10 ^e	282
5S NPEr	-79 +74	ATATCCCGGGAATTCGAGCTCGCCCCGG ATGCGCTAGCTAACAGGCCCGACCCCTGC	pXP10 ^e	139
5S NPEf	-79 +74	ATATGCTAGCAATTCGAGCTCGCCCCGG TATACCCGGGTAACAGGCCCGACCCCTGC	pXP10 ^e	153
SB2r	MCS 599	CTAGATCCCGGGCGACTCTAGAGGATCCCC CTAGCGGCTAGCACTCCGGGCGACACTTTG	pUC.SB2+ ^f	161

^a refers to the 5' end of each primer

^b restriction sites for cloning into the vacant DNR site are underlined. "0.5DNR1" used the DNR-flanking *NheI* site (nt # 4219) for the upstream primer. "0.5DNR2" used the DNR-flanking *XmaI* site (previously *XbaI* in pMCD, nt # 4454) for the downstream primer.

^c SV40 intron amplicon, Genbank Accession # U02434

^d Genbank accession # Y09885, positions relative to the *BamHI* site.

^e nt numbers are relative to the 5S RNA gene transcriptional start site.

^f nt numbers refer to Genbank accession #M12074 or the pUC18 multiple cloning site (MCS).

Table 2
PCR primer sequences for quantitation of nascent DNA

Primer	Sequence (5' to 3')	Position ^a
ppHyg	TGCTCCGCATTGGTCTTGA TGCGCCCAAGCTGCAT	797 ^b 869 ^b
pp2b	GCACTAGATGCTGAACCTAACAG CTGCCTTCATGCTGACATTTGTC	1184 ^c 1348 ^c
ppGlobin	AAACCCTGCTTATCTTAAACCAACC ACTCTGCCCTGCCTTTTATGC	54653 ^d 54727 ^d
pp8	CTCTCTCATAGTTCTCAGGC GTCCTCGGTATTAGTTCTCC	470 ^c 670 ^c
pp2	GTCCTGCCTCAAAACACAA CCTTCATGCTGACATTTGTC	1070 ^c 1348 ^c
pp6	AACTGGCTTCCCAAGAAATT AACCTCTGAAGTGAAGCTG	1517 ^c 1685 ^c
pp3	GGACACTAAGTCTAGGTACTACA GCTGGGATAAGTTGAAATCC	3882 ^c 4140 ^c

^a“Position” refers to the 5' end of each primer.

^b nt position relative to the Hygromycin resistance gene transcription start site

^c nt position relative to the BamHI recognition sequence in the DHFR ori-beta fragment of pMCD, defined as position 1 (Genbank accession # Y09885)

^d Refers to Genbank accession # U01317