Multiple Functional Elements Comprise a Mammalian Chromosomal Replicator

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The structure of replication origins in metazoa is only nominally similar to that in model organisms, such as Saccharomyces cerevisiae. By contrast to the compact origins of budding yeast, in metazoa multiple elements act as replication start sites or control replication efficiency. We first reported that replication forks diverge from an origin 5' to the human c-myc gene and that a 2.4-kb core fragment of the origin displays autonomous replicating sequence activity in plasmids and replicator activity at an ectopic chromosomal site. Here we have used clonal HeLa cell lines containing mutated c-myc origin constructs integrated at the same chromosomal location to identify elements important for DNA replication. Replication activity was measured before or after integration of the wild-type or mutated origins using PCR-based nascent DNA abundance assays. We find that deletions of several segments of the c-myc origin, including the DNA unwinding element and transcription factor binding sites, substantially reduced replicator activity, whereas deletion of the c-myc promoter P1 had only a modest effect. Substitution mutagenesis indicated that the sequence of the DNA unwinding element, rather than the spacing of flanking sequences, is critical. These results identify multiple functional elements essential for c-myc replicator activity.

The initiation of eukaryotic DNA synthesis is precisely regulated both at the G1/S phase checkpoint of the mitotic cycle and by mechanisms that control the order of replicon firing (reviewed in references 9, 18, and 21). For Escherichia coli, mammalian viruses, and the budding yeast Saccharomyces cerevisiae the initiation of DNA replication is controlled by trans-acting initiator proteins that interact with cis-acting DNA replicator sequences. For S. cerevisiae, replicators encompass 100 to 200 bp and include the major replication origin sites where DNA synthesis begins (8). These replicators contain an essential 11-bp autonomous replicating sequence (ARS) consensus sequence (ACS) that binds the origin recognition complex (ORC) to nucleate formation of prereplication complexes. By comparison, the domains that control replication in the fission yeast Schizosaccharomyces pombe are 500 to 1,500 bp in size and comprise multiple regions that add synergistically to origin activity (11, 22, 28, 48). The sequences that contribute to origin activity in S. pombe are heterogeneous, although A+T-rich potential binding sites for spORC have been found in several origins (10, 30, 36). Hence, despite notable differences in the structures of their respective ORC orthologs, the dispersed replication origins of metazoan cells more closely resemble those of S. pombe than those of S. cerevisiae in that multiple elements distributed over large distances act as replication start sites or control replication (3, 4, 13, 15, 16, 27, 40, 53, 56, 58, 62).

Replication begins in the 5' flanking region of the human c-myc gene, and a 2.4-kb core fragment of the c-myc origin displays plasmid ARS activity in transfected cells and in cell extracts (7, 15, 41-43, 56-58, 62). At the germ line locus, in plasmids, or at an ectopic chromosomal locus, replication initiates at multiple sites within the c-myc core origin and in the flanking DNA (15, 40, 58, 62). Thus, the c-myc core origin comprises both replicator elements and replication start sites. Among the structural elements of the 2.4-kb c-myc origin core are the P0 and P1 promoters, a number of transcription factor consensus binding sites, a DNA unwinding element (DUE) (also called the far upstream element [44]) which contains three 10-of-11 matches to the S. cerevisiae ARS consensus, a potential triple- or non-B DNA-forming region, a series of positioned nucleosomes (7, 33, 34, 54), and several DNA segments that adopt either bent or highly flexible conformations in vitro (7, 33, 34, 54). Similar sequences or structures are often found in the chromosomal replicators of S. cerevisiae and S. pombe. Their presence in the c-myc origin core raised the questions of which of these elements might contribute to replicator activity and whether the multiple initiation sites within and flanking the core origin are coordinately regulated.

In the present work we used the FLP recombinase system (40, 46) to build a series of clonal cell lines, each containing a mutated c-myc origin construct integrated at the same chromosomal location. Origin activity was defined by PCR quantitation of the abundance of short nascent DNA strands. Deletion of several different segments of the origin core, including the DNA unwinding element and transcription factor binding sites, substantially reduced chromosomal origin activity, whereas deletion of the c-myc promoter P1 had modest effect. Mutations that reduced origin activity did so at each of four sequence-tagged sites tested near the c-myc DNA. The results indicate that multiple functional elements are essential for c-myc origin activity and are consistent with prior indications that initiation sites flanking the core origin are subservient to the replicator activity of the core origin.

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Materials and methods

Cloning construction. Mutants of the wild-type c-myc origin core were constructed from the plasmid pFRT.myc (40) by overlapping PCR and standard cloning techniques. Numbering of c-myc sequences begins at the upstream HindIII cleavage site. In pFRT.myc.DUE the DNA unwinding element (nucleotides [nt] 747 to 828) was deleted. In plasmid pFRT.myc.DUE, nt 931 to 2359 were deleted. In pFRT.myc.DUE, 343 to 1420 both the DUE and the 3′-1,420 bp were deleted. A series of 12 to 200-bp deletions was constructed covering the c-myc core origin (pFRT.myc.D1 through pFRT.myc.D12); the endpoints of these deletions are given in Fig. 2. Plasmid pFRT.myc.Sub5AT was constructed by replacing the c-myc HindIII/Not core fragment with the 3′ flanking XhoI/EcoRV genomic c-myc fragment of nearly identical size. Plasmids pFRT.myc.Sub5AT and pFRT.myc.Sub5GC were constructed by replacing the 64% A+T 201 bp deletion DNA of pFRT.myc.ΔS with 201 bp containing 64% A+T or 64% G+C nucleotides, respectively (sequences available from the authors).

Cell culture, transfection, and selection. Acceptor 406 cells were derived from HeLa cells by integration of an FLP recombinase target (FRT) site (40) and maintained in Dulbecco’s modified Eagle medium with 10% newborn calf serum (Gibco-BRL) and 50 μg of gentamicin/ml in a humidified 5% CO2 atmosphere at 37°C. 406 cells were periodically reselected with hygromycin (400 μg/ml) to maintain Hgy-TK expression at the acceptor site. Transfections were performed using Lipofectamine 2000 (Gibco-BRL) following the procedure recommended by the manufacturer. For each transfaction the total amount of DNA was 1 μg, and the molar ratio of the donor plasmid and cotransfected FLP recombinase-expressing plasmid, pOG44, was 1:8. Approximately 24 to 48 h posttransfection, selection with 406.418 resistance (50 μg of active component/ml) was initiated and continued for 15 days. Single colonies were picked and exposed to 20 μM ganciclovir for 2 to 3 days. Colonies resistant to hygromycin, G418, and ganciclovir for 2 to 3 days. Colonies resistant to hygromycin, G418, and ganciclovir were applied to Hybond N+ membranes (Amersham) using a slot blot apparatus (27, 40). Briefly, approximately 7.5 × 10^6 cells were trypsinized 24 h after seeding and washed twice in cold phosphate-buffered saline. The cell pellet was resuspended in 240 μl of cold phosphate-buffered saline with 10% glycerol. Seventy-five microliters of cell suspension was loaded into one well of a 1.25% alkali low-melting-point agarose gel (SeaPlaque GTG) preheated to 4°C. Cells were lysed in the well for 10 min before the DNA marker was loaded and the current applied. The gel was run for 12 h at 40 V, neutralized in 1× Tris-acetate-EDTA (TAE) buffer (51), and stained in 1× TAE-0.5 μg of ethidium bromide/ml. The 1- to 2-kb nascent DNA was excised and purified (Qiagen).

Diagnostic PCR. Fifty nanograms of genomic DNA was used for PCR with primer sets designed for either the unoccupied chromosomal acceptor site or the acceptor site after integration of the donor plasmid (Table 1). PCRs contained 10 mM Tris-Cl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each of dGTP, dCTP, dATP, and dTTP, 1.25 U of Hotstar Taq DNA polymerase (Qiagen), and 25 pmol each of the forward and reverse primer for the following amplification regimen after an initial dwell at 94°C for 15 min: 94°C for 30 s, 55°C for 1 min, and 72°C for 3 min for a total of 30 cycles.

Southern blot hybridization. Analysis was performed using standard methods with 10 μg of genomic DNA and hybridizing with either the 756-bp EcoRI/XhoI fragment of the Hgy gene from plasmid pFRT.Hgy.TK or the 405-bp NcoI/XhoI fragment of the Neo gene from pFRT.myc, as previously described (40).

Slot blot quantitation. Nascent DNA or known quantities of genomic DNA were applied to Hybond N+ membranes (Amersham) using a slot blot apparatus (Schleicher and Schuell) (40) and UV cross-linked. Prehybridization and hybridization washes were as described for Southern blot hybridization, with 30 ng of [α-32P]dCTP randomly labeled genomic DNA used as a probe. The signals of the nascent samples were the genomic standards were quantified by PhosphorImage (Molecular Dynamics).

Quantitative PCR. Short (1 to 2 kb) nascent DNA was quantitated by competitive PCR (27, 40) using the primers and competitors shown in Table 1. The log of the ratio of signal intensity for the competitor and nascent template PCR products was plotted against the log of the competitor amount added to each reaction. GraphPad Prism was used to fit a straight line to the points, and the equation of this line was used to quantitate the amounts of nascent DNA template (12, 17, 40). Reaction conditions were selected to avoid heteroduplex formation between competitor and nascent PCR products, as evidenced by r^2 values near unity (29). To allow comparison of the results from PCRs of different nascent DNA preparations, the primary data from all reactions were normalized to an equivalent amount of input nascent DNA, based on slot blot quantitation of the total nascent DNA in each preparation (12, 17, 40). The data shown are representative of at least three independent nascent DNA preparations. Alternatively, quantitative real-time PCR (Q-PCR) was performed on an Applied Biosystems GeneAmp 5700 sequencer system with amplification monitored by SYBR Green fluorescence. The Q-PCR primers specific for the FRT acceptor site did not amplify HeLa genomic DNA. For Q-PCR the data were normalized against the signal from the low-nascent-strand abundance site STS-54.8 in the human b-globin locus (15, 27) amplified from an equal amount of the same nascent DNA in the same 96-well tray using the same reagents. The abundance of 1- to 2-kb nascent strands at STS-54.8 was roughly 40 copies/ng (based on Molecular Probes OligoGreen quantitation of total 1- to 2-kb nascent DNA). Standard curves were developed for all primer sets using known amounts of the same preparation of 406/pFRT.myc cell genomic DNA. The data presented are the means (and standard deviation) of triplicate analysis on at least three independent preparations of nascent DNA from each cell line.

Helical stability. Free energy values of wild-type c-myc DNA (nt 734 to 934) and the pFRT.myc.Sub5 substitution DNAs were calculated using WEFTHERMODYN software (http://wings.buffalo.edu/gsa/dna/dk/WEBTHERMODYN) (45) over 100-bp windows (1 bp per step, 10 μM monovalent ion, 37°C), and 20-bp windows (1 bp per step, 140 μM monovalent ion, 37°C).

Results

Selectivity of replicator activity. The chromatin around c-myc exon I comprises several nucleasie-hypersensitive sites that are stable to translocation (33, 34), a DNA unwinding element that is susceptible to in vivo to single-stranded-DNA-directed reagents (44), and a site of potential non-B DNA structure (Fig. 1a) (49). DNA replication initiates at the endogenous c-myc locus in the 5′ flanking region of the gene corresponding to the 2.4-kb HindIII/XhoI fragment and in the neighboring XhoI/EcoRV fragment (15, 56, 58, 62). The 2.4-kb c-myc origin HindIII/XhoI fragment displayed replicator activity when moved to the ectopic chromosomal acceptor site in the HeLa-derived cell line 406, but a bacterial sequence did not act as a replicator at the same site (40). To address the question of...
FIG. 1. Selectivity of c-myc origin replicator activity. (a) Map of the HindIII/EcoRV fragment of the human c-myc locus. The first c-myc exon and the 5' part of the second exon are indicated by filled boxes. The HindIII/XhoI fragment contains the wild-type c-myc origin core. The neighboring XhoI/EcoRV fragment used in pSub2.4 is also indicated. H, HindIII; N, NotI; Xh, XhoI; Ev, EcoRv. Downward-pointing arrowheads represent replication initiation sites previously mapped in vivo (15, 56, 58, 62). Filled squares and upward-pointing arrowheads designate sites hypersensitive to micrococcal nuclease or DNase I, respectively (6, 33, 34, 49, 54). A region of potential non-B, triplex structure is indicated by three horizontal bars. The diamond refers to a region of helical instability in vivo (DUE). (b) FLP recombinase integration system. 406 cells are the HeLa acceptor cell subline containing a single chromosomal copy of the plasmid pHyg.FRT.TK (linearized at its BglII sites), representing an unoccupied
whether another eukaryotic sequence placed at this acceptor site would act as a replicator, the XhoI/EcoRV fragment of c-myc DNA downstream of the origin core was substituted for the wild-type c-myc origin core in pFRT.myc to produce pFRT.myc.Sub2.4 and integrated into the same ectopic location in 406 cells using FLP recombinase (Fig. 1b) (40, 46). The targeted integration of all constructs was confirmed by analytical PCR using primers that generated diagnostic products from the empty or occupied acceptor sites and by Southern blot hybridization using probes complementary to the acceptor site (Hyg probe) or origin donor plasmid (Neo probe). As seen in Fig. 1c, diagnostic primers 1 and 2 yielded amplified product only with 406 cell DNA containing the unoccupied acceptor site, but not with DNA from 406/pFRT.myc or 406/pFRT.myc.Sub2.4 cells in which the acceptor site was occupied. Conversely, primers 1 and 3 yielded the expected amplified product only with DNA from 406/pFRT.myc or 406/pFRT.myc.Sub2.4 cells but not with 406 cell DNA. Hybridization with the Hyg probe confirmed that integration occurred at the intended chromosomal location, and hybridization with the Neo probe yielded a single hybridizing band, excluding the possibility that integration might also have taken place in a chromosomal location other than the acceptor FRT site (Fig. 1d).

Origin activity, as reflected by the abundance of short (1- to 2-kb) nascent DNA, was measured by Q-PCR. Other laboratories, as well as our own, have shown that this size fraction comprises new, semiconservatively synthesized DNA derived from replication origins and excludes Okazaki fragments (20, 60, 62). Similar approaches have been used previously to analyze the c-myc (40), DHFR (29), and β-globin (3, 27) origins. Q-PCR was performed at four sequence-tagged sites, STS-Hyg, STS-pVU, STS-pML, and STS-TK (Fig. 1b and e). Consistent with previous results using competitive PCR, integration of the wild-type c-myc core origin into the acceptor site of 406 cells significantly increased the nascent DNA abundance at all four sequence-tagged sites, with the greatest increases at the sites most closely flanking the c-myc origin DNA, STS-pVU and STS-pML. STS-pML and STS-pVU are 3 kb apart, and STS-Hyg and STS-TK are more than 8 kb apart, while the electrophoretically size-fractionated nascent DNA for these assays was no larger than 2 kb. Thus, the increases in nascent strand abundance at sequence-tagged sites STS-Hyg, -pVU, -pML, and -TK are consistent with our previous observations that the wild-type origin core DNA promotes independent initiations in the flanking chromosomal DNA. By contrast, pFRT.myc.Sub2.4 did not induce appreciable origin activity at the same chromosomal acceptor site. The relative abundance of 1- to 2-kb nascent DNA at STS-pVU or STS-pML in 406/pFRT.myc cells was about 10 to 15 times that in 406/pFRT.myc.Sub2.4 cells. These data confirm that the c-myc origin core displays replicator activity and argue that it is not merely the size or the eukaryotic derivation of the origin fragment that endows it with this property. Significantly, at the endogenous c-myc locus, replication initiates at several sites in the c-myc DNA contained in pFRT.myc.Sub2.4 (56, 58, 62). The absence of replicator activity in the XhoI/EcoRV fragment suggests that these initiation sites are subservient to the core origin.

cis-acting elements in the c-myc origin. The contribution to replicator activity of two regions of the origin, containing the DUE and the 3’ 1,420-bp transcription factor binding domain, were of particular interest since analogous elements have been implicated in origin activity in eukaryotic viruses and yeast, yet these elements are thought not to be functionally conserved in the replicators of higher eukaryotes. Plasmids were con-
constructed to remove an 82-bp region containing the DUE (pFRT.myc ΔDUE), or the 3’ 1,420-bp region containing multiple transcription factor consensus binding sites (pFRT.myc Δ3’ 1420) and integrated at the 406 cell acceptor site (Fig. 2). Targeted integration was confirmed by diagnostic PCR and Southern blot hybridization (Fig. 3a to c).

The origin activity of the wild-type and mutant origin constructs was compared at three sequence-tagged sites by competitive PCR (Fig. 4). Deletion of the DUE decreased the nascent strand abundance at STS-pML by approximately half and reduced the signals at the flanking sites STS-Hyg and STS-TK to background levels. Removal of the large region containing the transcription factor binding sites, in the presence or absence of the DUE, decreased the levels of nascent strands to background levels at all sequence-tagged sites, proximal and distal to the replicator.

These results raised the possibility that one or more transcription factor binding sites in the 3’ 1,420-bp region may contribute to origin activity. To assess the contribution of discrete subdomains within the c-myc origin to replicator activity, a set of 12 mutants, pFRT.myc Δ1-pFRT.myc Δ12, was constructed with ~200-bp deletions spanning the c-myc origin core (Fig. 2). Each construct was individually integrated into the same acceptor site in 406 cells using FLP recombinase (Fig. 3d and e). For comparison with subsequent real-time PCR quantitation of origin activity, competitive PCR was used initially to measure the activity of pFRT.myc Δ1 (Δ1) and pFRT.myc Δ10 (Δ10) relative to that of the wild-type c-myc DNA in pFRT.myc. The DNA deletion in pFRT.myc Δ1 removed one match to the S. cerevisiae ACS and a site of putative bent DNA, while the deletion in pFRT.myc Δ10 removed the F0 promoter (Fig. 5f). The data of Fig. 4b show that the pFRT.myc Δ1 deletion or the pFRT.myc Δ10 deletion reduced the abundance of nascent strands at STS-pML by ~70%. As in the case of the ΔDUE and Δ3’ 1420 deletions, Δ1 and Δ10 showed a reproducible but lesser decrease of nascent strand abundance in the flanking genome, with Δ1 reducing the signals by ~35% and ~50%, respectively, at STS-Hyg and STS-TK, and Δ10 reducing these signals by ~30 to 40%.

The dynamic range of Q-PCR is substantially greater than that of competitive PCR (2, 38). Thus, Q-PCR was used to assess the abundance of nascent strands at STS-Hyg, STS-pVU, STS-pML, and STS-TK in each of the 12 ~200-bp c-myc DNA deletion mutants, including Δ1 and Δ10 (Fig. 5). The results for different DNA preparations were normalized against the signal at STS-54.8 in the β-globin locus (chromosome 11). Normalizing the data from different deletion mutant cell lines against the nascent strand abundance at the lamin B2

406 cells containing wild type c-myc DNA (406/pFRT.myc) (W) or from cell lines 406/pFRT.myc ΔDUE (ΔDUE), 406/pFRT.myc Δ3’ 1420 (Δ3’ 1420), or 406/pFRT.myc ΔDUE, Δ3’ 1420 (ΔDUE, Δ3’ 1420) was digested with XhoI, electrophoresed, and hybridized to the Neo probe. The 5.8-kb band arose from the removal of an additional 1,420 bp from the 7.2-kb wild-type band. (c) Southern hybridization. Genomic DNA was isolated from 406 cells containing an unoccupied FRT (A), wild-type c-myc DNA (406/pFRT.myc) (W), or from the clonal cell lines 406/pFRT.myc Δ1 through 406/pFRT.myc Δ12 (numbered 1 to 12). DNA was digested with EcoRI, electrophoresed, and hybridized with the Hyg probe (upper panel) or the Neo probe (lower panel).
locus (chromosome 19) did not change the relative signals at the four acceptor site sequence-tagged sites. Thus, integration of c-myc DNA at the anonymous acceptor site in 406 cells does not appear to affect replication at these distal locations.

In 406 cells containing the wild-type c-myc origin DNA (406/pFRT.myc), Q-PCR revealed a difference of ~50-fold at STS-pVU or STS-pML versus STS-Hyg or STS-TK (Fig. 5b to e), whereas this difference was at most 8- to 15-fold using competitive PCR assays (Fig. 4). This difference in sensitivity may be because Q-PCR quantitation occurs at the earliest stages of amplification while competitive PCR is an end-stage assay, possibly more susceptible to spurious amplification products. As with the competitive PCR analyses, the results of the deletions are seen most clearly at those sites closest to the c-myc DNA, STS-pVU and STS-pML. The effects of the deletions in the cell lines 406/pFRT.myc.Δ1 and 406/pFRT.myc.Δ10 on nascent strand abundance are similar to, but greater than, those seen by competitive PCR. The relative abundances of nascent strands at the pVU and pML sequence-tagged sites in each deletion cell line are aligned above the position of that deletion in Fig. 5f.

Deletion of nucleotides 384 to 533 (Δ3) or nucleotides 1932 to 2133 (Δ11) had little or no effect on origin activity, whereas nascent strand abundance was most significantly decreased in Δ1 and Δ4–Δ10 and intermediate decreases were observed with the Δ2 and Δ12 cell lines. These data are consistent with the results obtained with the ΔDUE and Δ3′ 1420 cell lines, where removal of the DUE or multiple transcription factor binding sites reduced origin activity. While the greatest decreases correlate with the loss of specific structures in DNA or chromatin, the DUE, and matches to the ACS, there is no simple correlation with the absolute presence or absence of transcription factor binding sites. For example, Δ1 decreased origin activity but removed no known transcription factor consensus binding sites, while Δ3 removed a consensus binding site for c-Myb but did not decrease origin activity.

Since it was possible that the reduction of nascent strand abundance by deletions across the broad region defined by Δ4–Δ10 was due to a requirement for a particular spacing between segments 384 to 533 (Δ3) and 1932 to 2133 (Δ11) rather than the specific sequences in this region, two additional substitution cell lines were constructed that precisely restored the spacing of Δ5, which had removed the DUE and ARS consensus sites. The DNA deleted in 406/pFRT.myc.Δ5 is 64% A+T; this DNA was replaced with eukaryotic sequences of either 64% G+C or 64% A+T (Fig. 6a to c). As shown in Fig. 6d, substitution of either sequence did not restore the replicator activity of the c-myc origin fragment. These data indicate that the germ line spacing of the region between Δ3 and Δ11 is not sufficient for origin activity and further that the sequence of the DUE or ACS region is more important for origin activity than its base composition.

In S. cerevisiae a free energy of unwinding of less than 98 kcal/mole is associated with functional origins (45), and S. pombe ARSs meet this criterion as well, probably as a result of their A+T richness (28). As shown in Fig. 6e (100-bp window), both the 201-bp wild-type DNA corresponding to sequences missing from Δ5 and the Sub5AT DNA meet this criterion, while the Sub5GC DNA does not. A more obvious difference between wild-type and Sub5AT DNA is evident if the helical stability of 20-bp windows is considered (Fig. 6e, 20-bp window), where a local minimum appears between nucleotide positions 60 to 70 in wild-type DNA.

![FIG. 4. Analysis of nascent DNA abundance by competitive PCR.](http://mcb.asm.org/)

(a) Representative polyacrylamide gels of competitive PCR products with nascent DNA from the indicated cell lines. The products of amplification of nascent DNA (N) and competitor DNA (C) are indicated. The copy number of competitor added to each reaction is indicated above each gel lane. (b) Relative abundance of nascent DNA at the sequence-tagged sites STS-Hyg, STS-pML, and STS-TK. The amounts of nascent DNA template were calculated as described in Materials and Methods. To allow comparison of the results from PCRs with different amounts of input nascent DNA, the primary data (panel a) from all reactions were normalized to an equivalent amount of input nascent DNA, based on slot blot hybridization. Solid bar, wild-type c-myc (406/pFRT.myc); shaded bar, 406/pFRT.myc.ΔDUE; open bar, 406/pFRT.myc.Δ3′ 1420; hatched bar, 406/pFRT.myc.ΔDUE, Δ3′ 1420; stippled bar, Δ1 (406/pFRT.myc.Δ1); segmented bar, Δ10 (406/pFRT.myc.Δ10). The positions of the sequence-tagged sites on a map of the origin integrants are indicated below.
DISCUSSION

A 2.4-kb core fragment of the c-myc origin displays plasmid autonomous replicating sequence activity and replicator activity at an ectopic chromosomal location, while a bacterial DNA fragment did not (40). Together with the present results and data from others (4, 40), these results refute the contention that any similarly sized eukaryotic or prokaryotic DNA fragment will function as a replication origin in a chromosomal context.

FIG. 5. Origin activity of c-myc mutants at an ectopic chromosomal site. (a) Map of c-myc origin integrants showing sequence-tagged sites. (b to e) Q-PCR analysis of relative nascent DNA abundance at STS-Hyg (b), STS-TK (c), STS-pVU (d), and STS-pML (e). W, nascent strand abundance in cells containing wild-type c-myc DNA (pFRT.myc); 1 to 12, nascent strand abundance in cells containing mutant c-myc DNA (406/pFRT.myc.H9004 1-406/pFRT.myc.H9004 12). (f) Approximate location of structural features of the c-myc origin core DNA aligned with the positions of origin deletions (Δ1 to Δ12) described in this study. Horizontal bars, predicted positions of bent and straight or flexible DNA (34). MNase hss, micrococcal nuclease hypersensitive sites; DNase hss, DNase hypersensitive sites (33, 34, 54). DUE, DNA unwinding element (7, 44). Triplex, region of predicted triple helix or non-B DNA formation. ACS, 10 of 11 matches to S. cerevisiae ARS consensus sequence.txn factor, matches to transcription factor consensus binding sites: p, PuF (49); m, c-Myb (65); n, NF1 (54); s, SP1 (14); a, AP-2 (26); e, E2F (24, 50); f, far upstream element binding protein (44). P0 and P1, c-myc promoters.

FIG. 6. Sequence dependence of DUE region origin activity. (a) Maps of unoccupied (left) and occupied (right) acceptor sites. (b) Integration was verified by PCR. Diagnostic PCR using DNA from 406 acceptor cells (A) and DNA from cells containing a 201-bp A+T-rich (Sub5AT) or G+C-rich (Sub5GC) substitution in place of the wild-type 201-bp DUE/ACS region in pFRT.myc at the acceptor site. Other notations are as in Fig. 1. (c) Integran structure was verified by Southern hybridization with the Hyg or Neo probe to EcoRI-digested DNA from 406 acceptor cells (A), from 406 cells containing the wild-type c-myc origin fragment (406/pFRT.myc) (W), pFRT.myc.Sub5AT (Sub5AT), or pFRT.myc.Sub5GC (Sub5GC) at the acceptor site. (d) Relative nascent strand abundance at sequence-tagged sites STS-Hyg, STS-pVU, STS-pML, and STS-TK in 406 cells containing the wild-type c-myc origin fragment pFRT.myc (W), pFRT.myc.Sub5AT (AT), or pFRT.myc.Sub5GC (GC) at the acceptor site. (e) Helical stability calculations for the 201-bp DNAs corresponding to the wild-type c-myc DNA removed in deletion 5 (Δ5, nucleotides 734 to 934), Sub5AT DNA, and Sub5GC DNA. Left panel, 100-bp window. Horizontal gray bar, 98 kcal/mole. Right panel, 20-bp window. See Materials and Methods for details.
context. The notion that replication is sequence independent derives primarily from the replication of many DNAs in *Xenopus* oocyte extracts and of plasmids transfected into mammalian cells. In *Xenopus* oocyte extracts, origin-independent replication (23, 25, 39, 61) may be due to an elevated concentration of replication factors or the absence of a template chromatin structure restrictive to origin function (21, 35, 64).

It has been reported that any prokaryotic or eukaryotic DNA sequence of sufficient length can initiate replication in plasmids that bind the viral EBNA-1 protein (32). The present data argue against concluding from this that specific DNA sequences are not involved in chromosomal replicator activity. In mammalian cells as in yeast, plasmid replication may be more permissive than chromosomal replication. Thus, the 5’ 930-bp of the c-myc core display autonomous replicating sequence activity (43), but not chromosomal replicator function (this work). Moreover, the nuclear retention function ascribed to EBNA-1 binding may be related to the attachment of cellular origin recognition complex proteins and prereplicative complexes (15, 52) that act in cis to potentiate the initiation of replication in DNA sequences sufficiently long to unwind under superhelical strain that otherwise would not function as chromosomal replicators or origins.

The c-myc 2.4-kb core origin fragment stimulates replication in cis. When integrated into the same acceptor site, the origin fragment acted as a replicator while the Sub2.4 fragment did not, although replication initiates within the Sub2.4 fragment at its endogenous chromosomal location. The simplest interpretation of these results is that the core origin stimulates replication in the flanking DNA when moved to the ectopic acceptor site in the neighboring Sub2.4 *XhoI/EcoRV* DNA at the endogenous c-myc locus. Recent experiments with *Drosophila* (5) and *S. pombe* (31) predict that ORC binding to specific regions of DNA is involved in metazoan replicator function. Hence, it is reasonable to speculate that one or more of the regions deleted in Δ5-Δ12 acts to recruit ORC or other replication proteins (e.g., MCMs) to the neighboring Sub2.4 DNA (5, 19) at the c-myc locus. However, at this time it is not known whether ORC is bound to the wild-type or Δ1 to Δ12 deletion mutants.

The replicators of *S. cerevisiae* are compact, while those of *S. pombe* are broad (11, 28, 48) and contain multiple consensus sequences, ORC binding sites, and transcription factor binding sites. In the present work several mutations of the c-myc core origin decreased c-myc replication initiation, including deletion of the ACS or DUE sequences, transcription factor consensus binding sites, potential triplex or non-B DNA structures, and DNA bound as positioned nucleosomes. Nonetheless, deletion analysis by itself does not reveal the positive role in replication of those elements whose removal decreased the activity of the c-myc replicator.

Alterations in nucleosome positioning are correlated with changes in the binding of replication proteins and origin activity (37, 55). At the endogenous c-myc locus and at ectopic locations the ~1.4-kb zone defined by pFRT.myc.Δ4-pFRT. myc.Δ10 contains several specific MNase/DNaseI cleavages indicative of a nonrandom chromatin organization. This chromatin structure is stable to transduction to ectopic chromosomal sites (33, 34). The present results show that any of the deletions within this zone substantially reduced origin activity. To test whether the particular spacing of sequences in this zone is essential, the DUE fragment deleted in Δ5 was replaced with A+T-rich or G+C-rich DNA of the same length. Although these substitutions restored the spacing of the core origin, they did not restore replicator activity, suggesting that at least in the case of the DUE or ACS element specific sequences or structures may be important for replicator activity. Proof that the decrease of nascent strand abundance in the remaining deletions is due to the loss of specific sequences will require analysis of additional substitution mutant cell lines.

Compared to the acceptor 406 cells or 406/pFRT.myc.Sub2.4 cells, integration of the wild-type c-myc origin core at the acceptor site increased the abundance of 1- to 2-kb nascent strands at STS-pVU and STS-pML, which are 3 kb apart, and at STS-Hyg and STS-TK, which are more than 8 kb apart. Moreover, except for the Δ2 and Δ3 cell lines, the effects of the remaining c-myc origin deletions on nascent strand abundance were qualitatively similar at all four sequence-tagged sites, although quantitatively greater at the proximal STS-pVU and STS-pML than at the distal sequence-tagged sites. While it is formally possible that the nascent strands detected at the distal sites are 3- to 8-kb contaminants in the 1- to 2-kb nascent fraction and therefore the same effects will be seen at all four sequence-tagged sites, the results from Δ2 and Δ3 argue against this. The disparate effects of Δ2 or Δ3 at the proximal versus distal sequence-tagged sites may reflect different influences on individual initiation sites, albeit for unknown reasons. In control experiments (not shown), nascent DNA preparations were spiked with fluorescently labeled DNA of known size, prior to alkaline gel fractionation. These experiments detected no cross-contamination between the 1- to 2-kb and 3- to 8-kb fractions of DNA. Taken together, the results are consistent with previous observations that the c-myc replicator promotes initiation at multiple sites (40, 56, 58, 62). By contrast, for those *S. pombe* and human origins that have been examined by replication initiation point mapping, a single site appears to be preferred as the leading strand start in the locale of the replicator (1, 22, 31), with additional initiations in the flanking DNA revealed by two-dimensional electrophoresis (22). Therefore, it is possible that at the ectopic acceptor locus in 406 cells, the relative nascent strand abundance at STS-pVU or STS-pML versus that at STS-Hyg or STS-TK may reflect the difference between the frequency of initiation at primary and flanking sites.

The ACS of *S. cerevisiae* origins is not functionally conserved in *S. pombe*, and a recent report showing that the function of the A+T-rich B2 element did not correlate with local helical instability (63) contests earlier results showing that origin efficiency is related to the ease of origin unwinding (59). These data challenge the significance of the c-myc DUE region as an unwinding element. Some form of DNA unwinding is an obligatory step for initiation of replication, and the c-myc DUE region exists in vivo in a non-base-paired conformation (44). Our experiments have shown that replacing the easily unwound DUE with a sequence of equivalent A+T content does not allow c-myc replicator function. At the same time, these results do not allow conclusions about the role of an easily unwound region in the c-myc origin, since the Sub5AT replacement did not show the same local unwinding profile as the wild-type DUE and because a potential protein binding site
may have been eliminated by the substitutions. In this context it is of interest to note other work, in which members of our group have used a yeast one-hybrid strategy to isolate a novel protein of the predicted molecular mass 24 kDa, which binds specifically to the DUE region in vivo and inhibits replication subsequent to the formation of prereplicative complexes on sperm chromatin in vitro in the *Xenopus* oocyte extract system (J. Casper, M. Ghosh, G. Randall, and M. Leffak, unpublished data). Experiments are under way to address the role of the c-myc DUE in replicator function by replacing it with other sequences of predicted low helical stability.

The pFRT.mycΔ3' 1420 mutant removed several transcription factor binding sites and eliminated origin activity, as did smaller deletions of particular transcription factor consensus binding sequences (Δ4–Δ10), while deletion of other consensus binding sites had a modest effect or no effect (Δ2, Δ3, Δ12), raising the question of whether transcription factor binding sites contribute to origin activity. Possibly relevant to this question are experiments by Obha et al., who found that DNA replication from the c-myc core origin in vitro was stimulated by transcription (47). Experiments are in progress to test the effect on replication of replacing the c-myc transcription factor binding sites with heterologous protein binding sites.

The human c-myc core origin shows incomplete similarity to the origin of *S. cerevisiae* or *S. pombe*. While the c-myc origin contains a DUE, ARS consensus sequences, and transcription factor binding sites, these elements occupy a broader region than the compact origins of *S. cerevisiae*. Whereas the origins of *S. pombe* are broad and contain multiple asymmetric A+T-rich elements that contribute incrementally to plasmid ARS activity, several essential elements of the c-myc origin do not contain asymmetric A+T sequences, and replacement of the A+T-rich DUE region with an equally A+T-rich DNA sequence did not restore c-myc chromosomal origin activity. In summary, the present work shows that diverse DNA elements contribute to the activity of the human c-myc replicator. Although deletion of the DUE region and several transcription factor consensus binding sites reduced nascent strand abundance, these experiments do not identify the roles of these elements in replication initiation. Proof of how these elements contribute to replication will involve their replacement with other sequences that restore activity.

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REFERENCES


