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Activation of a human chromosomal replication origin by protein tethering

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ABSTRACT

The specification of mammalian chromosomal replication origins is incompletely understood. To analyze the assembly and activation of prereplicative complexes (pre-RCs), we tested the effects of tethered binding of chromatin acetyltransferases and replication proteins on chromosomal c-myc origin deletion mutants containing a GAL4-binding cassette. GAL4^{DBD} (DNA binding domain) fusions with Orc2, Cdt1, E2F1 or HBO1 coordinated the recruitment of the Mcm7 helicase subunit, the DNA unwinding element (DUE)-binding protein DUE-B and the minichromosome maintenance (MCM) helicase activator Cdc45 to the replicator, and restored origin activity. In contrast, replication protein binding and origin activity were not stimulated by fusion protein binding in the absence of flanking c-myc DNA. Substitution of the GAL4-binding site for the c-myc replicator DUE allowed Orc2 and Mcm7 binding, but eliminated origin activity, indicating that the DUE is essential for pre-RC activation. Additionally, tethering of DUE-B was not sufficient to recruit Cdc45 or activate pre-RCs formed in the absence of a DUE. These results show directly in a chromosomal background that chromatin acetylation, Orc2 or Cdt1 suffice to recruit all downstream replication initiation activities to a prospective origin, and that chromosomal origin activity requires singular DNA sequences.

INTRODUCTION

The bacterial replicon model proposed that *cis* acting replicator DNA elements are bound by *trans* acting initiator proteins to activate the replication of flanking DNA (1). The goal of the present experiments is to identify DNA elements and initiator proteins responsible for initiating mammalian chromosome replication. The bacterial model applies generally in yeast, where binding of the origin recognition complex (ORC) initiates replisome assembly at replication origins of *Saccharomyces cerevisiae* plasmids and chromosomal DNA [reviewed in (2)]. Similarly, the replication origins of *Schizosaccharomyces pombe* and metazoans contain binding sites for the orthologous ORC proteins, typically near an easily unwound AT-rich DNA unwinding element (DUE). While *S.pombe* Orc4 has been shown to recognize AT tracts (3–5), aside from preferential binding to negatively supercoiled DNA, metazoan ORCs show little sequence selectivity (6,7), and no consensus DNA sequence has been shown to possess metazoan chromosome replicator activity.

ORC binding to chromatin promotes the recruitment of Cdc6/Cdc18, Cdt1 and the minichromosome maintenance (MCM) proteins to form prereplicative complexes (pre-RCs) at origins (8). However, pre-RC formation does not unavoidably lead to replication initiation (9–13). Thus, the ORC and MCM complex are bound at the presumptive ATXN10/SCA10 (Gene ID 25814) origin, and to a DUE deletion mutant of the c-myc origin, but DUE-binding protein (DUE-B), Cdc45 or RPA binding, and origin firing do not occur unless a DUE of sufficient length is present (13,14). Therefore, it is not known whether a DUE and DUE-B binding are both required for replication initiation.

During an unperturbed cell cycle, activation of the pre-RC to the preinitiation complex (pre-IC) by S-phase CDK and Cdc7 kinase activities involves the stable loading of the helicase activator Cdc45, and the Go-Ichi-Ni-San; Psf1, Psf2, Psf3, Sld5 (GINS) proteins to form the replisome Cdc45-MCM-GINS (CMG) complex (15–17). Three additional proteins, Treslin/TICRR (18,19), GemC1 (20) and DUE-B (13,21,22), have been shown to interact with TopBP1 and are involved in the loading of Cdc45, but the molecular details of the Cdc45 loading reaction are unclear (23–25), and the difference between a pre-RC that will become activated and one that will not is currently uncertain.

Relevant to the issue of origin activation by protein binding, targeting of GAL4^{DBD}-Orc1, GAL4^{DBD}-Orc2 or GAL4^{DBD}-Cdc6 fusion proteins to tandem

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GAL4-binding sites has been reported to induce plasmid replication in human transformed cells (26). Because the factors necessary to initiate origin activity in a plasmid versus chromosomal environment may differ (27–30), another recent study examined *Drosophila* S2 cells containing multiple randomly integrated chromosomal copies of the upstream activation sequence (UAS) cassette, each comprising five GAL4-binding sites. In this system expression of $GAL4^{DBD}$-Orc5 fusion proteins could induce cell cycle-, pre-RC-dependent replication (31). However, tethering of $Gal4^{DBD}$-Orc5 or $GAL4^{DBD}$-Cdc6 did not elicit detectable plasmid replication in S2 cells (31). Differences in tissue type, transformed human versus nontransformed *Drosophila* cells, fusion protein expression or plasmid versus chromosomal environments may explain this disparity.

The HBO1 acetyltransferase interacts with the Orc1, Cdt1 and Mcm2 pre-RC proteins, and chromatin acetylation has been linked to the stimulation of replication origin activity. Treatment with the histone deacetylase inhibitor trichostatin A altered the timing and global patterns of MCM binding and replication initiation in human cells (32). Origin binding of Cdt1 and recruitment of HBO1 results in chromatin decondensation (33,34), with histone H4 K5, K8, K12 acetylation marking nucleosome packing accessible for pre-RC formation (33,35–38). Geminin repression of the histone acetyltransferase activity of the Cdt1/HBO1 complex, or expression of the enzymatically inactive $HBO1^{G485A}$ mutant correlated with decreases of global histone H4 acetylation and origin activity (35). Tethering of the *Drosophila* HBO1 ortholog (*Chameau*) to the minimal third chorion origin significantly stimulated local replication initiation (39). In the present work, we have characterized the effect of chromatin acetylation on origin activity and replication protein loading at a human origin.

We have modeled the effect of chromatin acetylation, protein binding and DNA structure on a specific replication origin using an ectopically integrated single copy of the human c-myc origin replicator. The ectopic c-myc replicator displays the replication efficiency, bidirectional polarity, replication timing, nucleosome positioning and chromatin structure of the endogenous c-myc origin (32,38,40). As assessed by chromatin immunoprecipitation (ChIP), the binding of the ORC, MCM, Cdc45 and DUE-B replication proteins is similar in amount and position to that at the endogenous c-myc origin (13,14,38,41). To target the effects of protein binding and DNA structure specifically to the c-myc origin without affecting initiation at other origins, the 2.4 kb c-myc replicator was inactivated by replacement of the 3′ ~1.4 kb of c-myc DNA with a cassette containing five tandem GAL4-binding sites (5× GAL4). We examined the effects of tethering GAL4 DNA–binding domain ($GAL4^{DBD}$) protein fusions with the replication proteins HBO1, Orc2, Cdt1, Mcm7 or Cdc45 to the inactivated c-myc replicator. We also examined the effect of targeting the E2F1 transcription factor to the inactivated c-myc origin. E2F1-3 family members have been implicated in replication activation through association with histone acetyltransferases (42,43), and E2F1-binding sites have been identified flanking the c-myc origin (44–46). Our results indicate that replication proteins were recruited to the inactivated c-myc origin, and origin activity was restored, following local chromatin acetylation. These effects were dependent on the presence of c-myc origin DNA, and show that sequences in the 5′ region of the c-myc origin act in concert with chromatin proteins to induce origin activity.

To assess whether the c-myc 5′ DUE is necessary for origin activity apart from its binding of DUE-B, we asked whether DUE-B binding is sufficient for origin activation by substituting the GAL4-binding site cassette for the c-myc DUE. It was previously shown that a scrambled c-myc DUE sequence inactivated the c-myc replicator, but that DUE-B and Cdc45 binding, and ectopic site origin activity, could be restored by heterologous sequences comprising $(ATTCT)_{n\geq 27}$ repeats from the ATXN10/SCA10 locus (13,14). Irrespective of binding of the $GAL4^{DBD}$-DUE-B fusion protein, substitution of the GAL4-binding site for the DUE inactivated the origin, showing that DUE-B binding at the location of the DUE is not sufficient for origin activation. ChIP results suggest that a singular feature of DUE structure is necessary for the recruitment of Cdc45 to the pre-RC.

MATERIALS AND METHODS

Cell culture and transfection

HeLa and HeLa/406 derivative cell lines containing the c-myc replicator integrated at the ectopic FLP recombinase target site (40,41,47,48) were cultured in Dulbecco's modified Eagle's medium with 10% newborn calf serum in a 5% CO_2 atmosphere. Clonal HeLa/406 cell lines were selected with 400 μg/ml hygromycin, 600 μg/ml G418 or 20 uM ganciclovir (GCV) as described (40). Lipofectamine 2000 (Invitrogen) was used for plasmid DNA transfection as recommended by the manufacturer. Approximately 1.5×10^7 cells per 15 cm plate were transfected with 50 μg GAL4 plasmid plus 5 μg GFP internal transfection control plasmid. Cells were split after 16 h and harvested 24 h later. All cell lines used in this article are listed in Figure 1; HeLa/FRT.myc (48), HeLa/FRT.myc.5′(930), HeLa/FRT.myc.5′(930)-GAL4 and HeLa-GAL4, were transfected by $GAL4^{DBD}$ fusion protein expression plasmids as described (37).

Plasmids expressing $GAL4^{DBD}$ fusion proteins

$GAL4^{DBD}$ fusions with human replication proteins are diagrammed in Figure 1. Plasmid pBS $GAL4^{DBD}$-E2F1 was a generous gift from Dr Erik Flemington (49). Other cDNAs were generous gifts of Dr Masayoshi Iizuka [HBO1, $HBO1^{G485A}$ (50)], Dr Anindya Dutta [Orc2 (26)], Dr Iwao Kukimoto [Cdc45 (51)] and Dr Hideo Nishitani [Cdt1 (52)]. These cDNAs, as well as Mcm7 and DUE-B cDNAs (cloned from a human cDNA library) were fused to the GAL4 DNA binding domain ($GAL4^{DBD}$, amino acids 1–147), and replaced the EGFP cDNA in pEGFP N1 (Clontech), except that $GAL4^{DBD}$-Cdt1 was expressed in pcDNA3.1 (Invitrogen).

All GAL4^{DBD} fusion protein constructs were confirmed by DNA sequencing.

Quantitative polymerase chain reaction

Nascent DNA (0.6–2 kb) was fractionated by one-step denaturing gel electrophoresis (40,41,48,53). Approximately 5×10^6 cells were loaded in each of four wells of an alkaline agarose gel for nascent DNA isolation [Qiagen Gel Extraction kit (14,40,47,54)]. For nascent DNA abundance analysis, the β-globin STS-54.8 was used as an internal normalizer (53). ChIP was carried out as previously described (32,38) except that a ChIP DNA Clean & Concentrator kit (Zymo Research) was used to purify the reverse cross-linked DNA for real-time quantitative polymerase chain reaction (PCR) analysis. Quantitative real-time PCR (qPCR) was performed on the ABI Prism 5700 or Prism 7000 Sequence Detection Systems. PCR reagents were products of Applied Biosystems. Absolute DNA copy number was quantitated using standard curves generated for each set with known amounts of sheared genomic DNA. ChIP values are the enrichment relative to preimmune IgG immunoprecipitations. All experiments are the results of at least two chromatin isolations, two independent immunoprecipitations and triplicate qPCRs. PCR primer sequences used in this study have been published (38,47).

Western blotting

Protein expression in cell lysates was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis as described (37). Anti-acetylated histone H4 antibody was a product of Upstate Biotechnology. Polyclonal antibodies against human Orc2 and Mcm7 used in some western blots were obtained from Dr Aloys Schepers. Anti-DUE-B antibody was raised in rabbits against recombinant human DUE-B expressed in bacteria. Anti-E2F1 antibody and anti-Cdc45 antibody were purchased from Santa Cruz Biotechnology. Anti-GAL4^{DBD} and anti-HBO1 antibody were from Abcam.

RESULTS

Constructs

The c-myc replicator constructs were integrated at the same unique chromosomal FRT acceptor site (chromosome 18) in clonal cell lines derived from HeLa/406 cells (40,41,47,48) as shown in Figure 1. Clonal cell lines are named for their respective plasmid integrants, thus HeLa/FRT.myc cells contain the wild type 2.4 kb core c-myc replicator in the integrated plasmid pFRT.myc (Figure 1a). Figures 1b and c, respectively, summarize our previous analyses of the effects of ~200 bp deletions on ectopic c-myc origin activity (40), and the pattern of replication protein binding across the c-myc origin (38). As shown in Figure 1d, HeLa/FRT.myc.5′(930)-GAL4 cells contain the 5′ 930 bp of the c-myc replicator (including the c-myc DUE) flanked by the 5× GAL4-binding site cassette; HeLa/FRT.myc.5′(930) cells contain the 5′ 930 bp of the c-myc replicator without the 5× GAL4-binding site cassette; HeLa/FRT.GAL4 cells contain the 5× GAL4-binding site cassette without flanking c-myc sequences; HeLa/FRT.myc.subDUE cells contain the 2.4 kb core c-myc replicator in which the DUE has been replaced by the 5× GAL4-binding site cassette, and HeLa/FRT.myc.ΔDUE cells contain the 2.4 kb core c-myc replicator minus the DUE (14,40). Nascent DNA derived at the ectopic site integrants can be distinguished from nascent DNA at the endogenous c-myc origin using the qPCR primers STS-pVU and STS-pVD. The GAL4^{DBD} fusion proteins used in this work are diagrammed in Figure 1e.

Native binding of GAL4^{DBD} proteins

To assess their native conformation and origin binding ability, the GAL4^{DBD} fusion proteins were expressed in HeLa cells, and ChIP was used to assay protein binding to the wild-type c-myc replicator using qPCR primers STS-5′, -A, -C and –E, which are specific to the endogenous c-myc locus (Supplementary Figure S1). Consistent with previous results, the E2F1 fusion protein was most strongly enriched near STS-E (relative to the distal primer site STS-5′); the Mcm7, DUE-B and Cdc45 fusion proteins were most highly enriched near the DUE (STS-C), and the Orc2, HBO1, and Cdt1 fusion proteins co-localized at two sites, near STS-A and STS-E (13,38,45,55–57). In contrast, the GAL4^{DBD} alone did not show origin binding above background levels. These results suggest that the conformations required for protein and DNA interaction in pre-RC and pre-IC assembly are preserved in the GAL4^{DBD} fusion proteins.

Binding of *trans* acting factors at the ectopic c-myc replicator

The 2.4 kb core fragment of c-myc replication origin is a *cis* acting replicator when integrated at an ectopic chromosomal site in HeLa cells (48), while mutant constructs of the c-myc origin do not initiate replication at this ectopic chromosomal site (Supplementary Figure S2). Similarly, whereas endogenous Orc2, Cdt1, HBO1, Mcm7, DUE-B and Cdc45 bound to the wild-type ectopic c-myc replicator, these proteins (or the GAL4^{DBD}) did not bind appreciably to the c-myc origin mutant constructs (Supplementary Figure S2). In contrast, we could not detect binding of E2F1 within the ectopic c-myc replicators regardless of the origin activity of the wild-type or mutant c-myc replicator cell lines, despite a strong E2F1 signal near the 3′ end of the endogenous c-myc core replicator. This is consistent with previous reports of E2F1 binding within ~40 bp downstream of the c-myc core replicator (44–46,58). Independent experiments have also shown that the 2.4 kb c-myc core replicator at this ectopic site displays the replication timing, chromatin structure, replication protein binding and origin activity of the endogenous c-myc origin (13,32,38). Together, these results confirm that functional replication complexes assemble at the ectopic c-myc replicator.

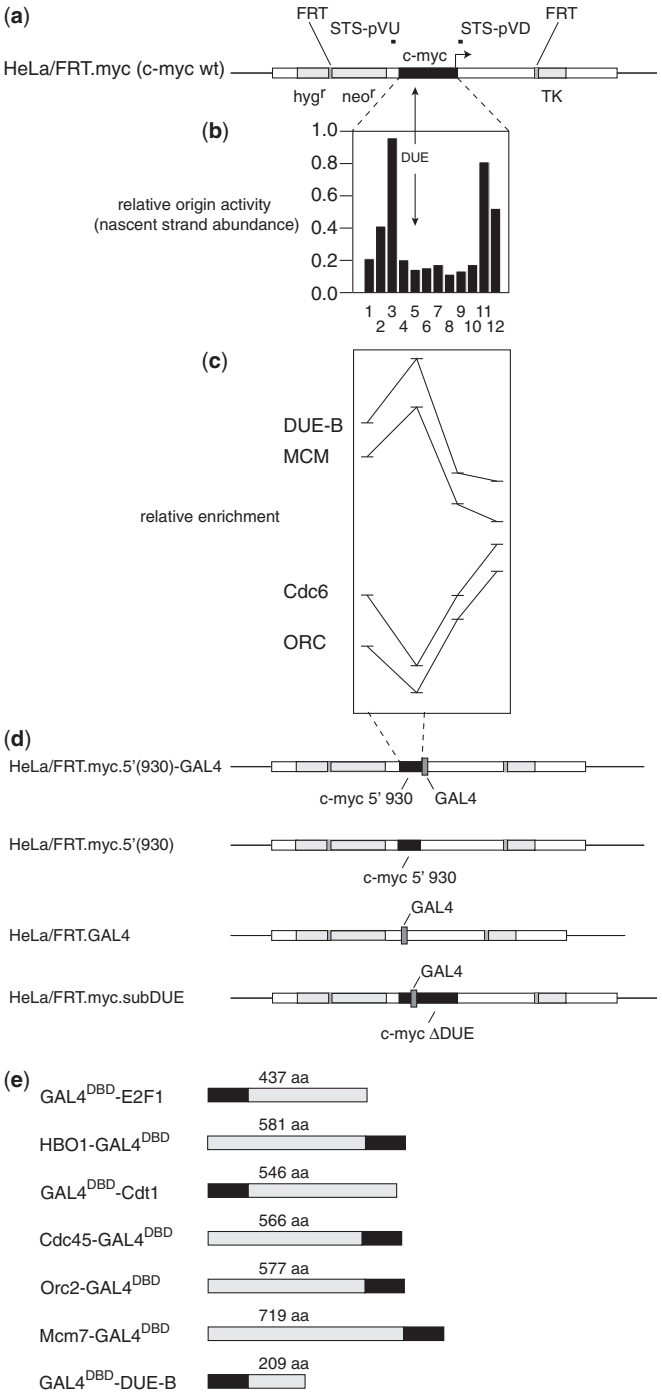

Figure 1. Constructs used in this study. (**a**) Wild-type c-myc origin integration at the ectopic Flp recombinase target (FRT) site in a clonal cell line. HeLa/FRT.myc contains the wild-type 2.4 kb c-myc replicator core (40). Bent arrow, c-myc Po promoter. (**b**) Origin activity (nascent strand abundance at STS-pVU) of ~200 bp deletion mutants (1–12) of the 2.4 kb c-myc replicator integrated at the ectopic FRT (relative to the intact c-myc 2.4 kb replicator at the FRT) (40). The DUE is absent in deletion 5. (**c**) Relative enrichment (ChIP) of replication proteins at four sequence tagged sites (horizontal bars) across the c-myc replicator (38). The highest value for each line is set at 100%. (**d**) c-myc origin mutants integrated at the ectopic FRT site. HeLa/FRT.myc.5′(930)-GAL4 contains the GAL4-binding site cassette alongside the 5′ 930 bp of the c-myc replicator; HeLa/FRT.myc.5′(930) contains the 5′ 930 bp of the c-myc replicator; HeLa/FRT.GAL4 contains the GAL4-binding site cassette; HeLa/FRT.myc.GAL4sub DUE contains the 2.4 kb c-myc replicator in which the ~200-bp DUE domain (40) has been replaced by the GAL4-binding cassette. FRT, Flp recombinase target; hygr, hygromycin resistance gene; neor, G418 resistance gene; TK, HSV thymidine kinase gene; GAL4, 5 × GAL4-binding site cassette; STS-pVU, STS-pVD, PCR primer binding sites; black bar, c-myc replicator DNA. (**e**) Diagrams of replication $GAL4^{DBD}$ fusions with replication proteins. Black bar, $GAL4^{DBD}$.

Tethered E2F1 binding restored origin activity

$GAL4^{DBD}$-E2F1 was expressed at a level similar to that of endogenous E2F1 following transfection (Figure 2a), and could restore origin activity to the inactivated FRT.myc.5′(930)-GAL4 replicator equivalent to the origin activity of the wild-type core replicator (Figure 2b), dependent on the presence of flanking c-myc origin DNA. ChIP results indicate that the active 5′ 930 bp c-myc replicator domain resides within acetylated chromatin (Figure 2c). However, the replicator inactive FRT.GAL4 (Figure 2b) is also within acetylated chromatin (Figure 2c), demonstrating that local chromatin acetylation is not sufficient to induce origin activity. These results also indicate that the 5′ 930 bp of c-myc replicator DNA can promote origin activity within acetylated chromatin.

$GAL4^{DBD}$ ChIP showed that $GAL4^{DBD}$-E2F1 binding was dependent on the presence of the GAL4-binding site (Figure 2d), and led to the recruitment of endogenous Orc2, Mcm7, DUE-B and Cdc45 preferentially to the FRT.myc.5′(930)-GAL4 origin (Figure 2e-h). Although $GAL4^{DBD}$-E2F1 also bound to the GAL4-binding site in FRT.GAL4 cells (Figure 2d), and despite a modest enrichment for Orc2 binding in these cells (Figure 2e), Mcm7, DUE-B and Cdc45 were most strongly recruited only to the GAL4-binding site flanked by c-myc DNA, suggesting that recruitment of pre-RC and pre-IC components by ORC, and origin activation, are enhanced by DNA sequences in the 5′ domain of the c-myc replicator.

Tethered HBO1 acetyltransferase activity restored origin activity

Tethering of the *Drosophila* HBO1 ortholog at the third chorion origin significantly stimulated replication initiation (39). To test directly whether acetyltransferase activity could stimulate the c-myc replicator, $GAL4^{DBD}$ fusions with wild-type HBO1 or the catalytically inactive $HBO1^{G485A}$ mutant were expressed in cells containing the ectopic GAL4-binding site (Figure 3a). Wild-type HBO1, but not the $HBO1^{G485A}$ mutant, selectively stimulated origin activity at the GAL4-binding site flanked by c-myc replicator DNA (Figure 3b), although both proteins bound equally well to the GAL4-binding sites regardless of c-myc flanking DNA (Figure 3c). As expected, histone acetylation was enhanced only by the wild-type HBO1 fusion protein (Figure 3d). Parallel to the stimulation of origin activity, Orc2, Mcm7, DUE-B and Cdc45 were selectively recruited to the c-myc-GAL4–binding site by wild-type HBO1 (Figure 3e-h), strengthening the conclusion that chromatin acetylation acts synergistically with one or more singular DNA

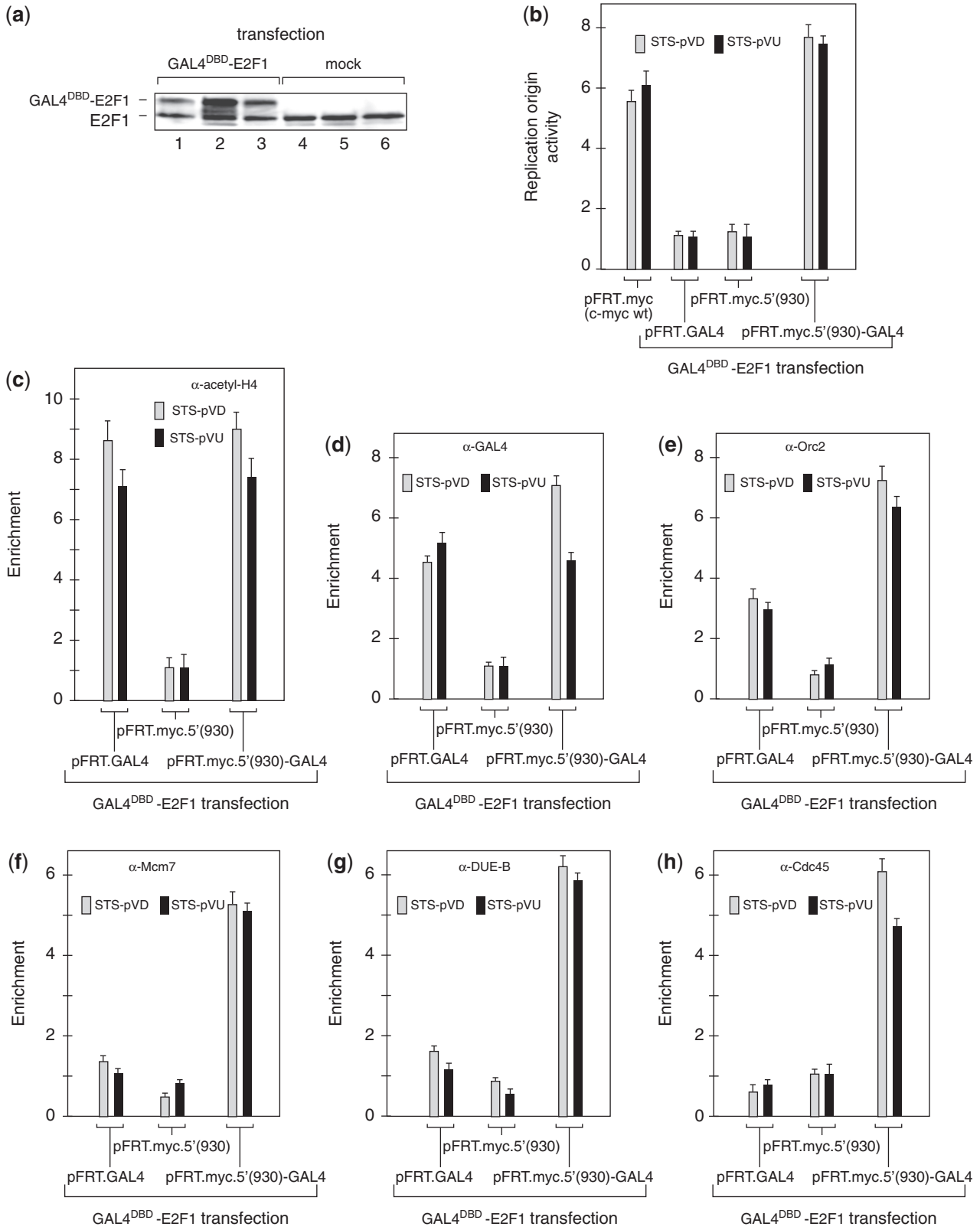

Figure 2. GAL4^{DBD}-E2F1 induces origin activity. (**a**) Western blot with anti-E2F1 antibody of extracts from cells containing (lanes 1, 4) FRT.GAL4, (lanes 2, 5) FRT.myc.5′(930), (lanes 3, 6) FRT.myc.5′(930)-GAL4. Lanes 1-3, cells expressing GAL4^{DBD}-E2F1; lanes 4-6, cells transfected with vector only. (**b**) Replication origin activity measured as nascent strand abundance at STS-pVU and STS-pVD in cells containing FRT.myc (WT), or cells expressing GAL4^{DBD}-E2F1 and containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4. (**c**-**h**) ChIP using the indicated antibody (α) was quantitated by qPCR at STS-pVU and STS-pVD in cells containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4.

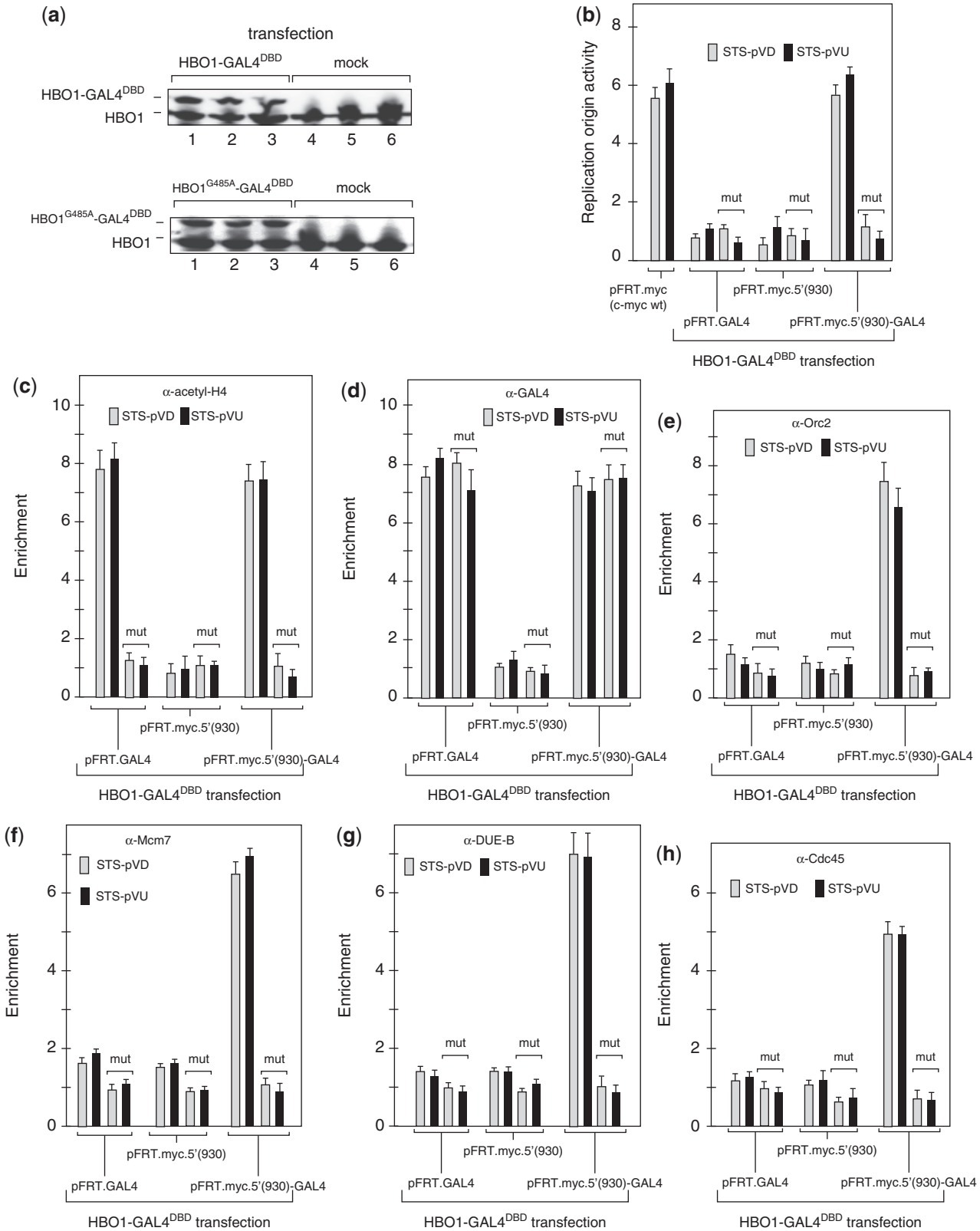

Figure 3. HBO1-GAL4^{DBD} induces origin activity. (**a**) Western blot with anti-HBO1 antibody of extracts from cells containing (lanes 1, 4) FRT.GAL4, (lanes 2, 5) FRT.myc.5′(930), (lanes 3, 6) FRT.myc.5′(930)-GAL4. (Upper) Lanes 1-3, cells expressing HBO1-GAL4^{DBD}; lanes 4-6, cells transfected with vector only. (Lower) Lanes 1-3, cells expressing HBO1^{G485A}-GAL4^{DBD} (mutant, mut); lanes 4-6, cells transfected with vector only. (**b**) Replication origin activity measured as nascent strand abundance at STS-pVU and STS-pVD in cells containing FRT.myc (WT), or cells expressing HBO1-GAL4^{DBD} or HBO1^{G485A}-GAL4^{DBD} and containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4. (**c-h**) ChIP using the indicated antibody (α) was quantitated by qPCR at STS-pVU and STS-pVD in cells containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4.

structures in the 5′ 930 bp of the c-myc replicator to promote replication protein binding and origin activity. These results suggest also that ORC-binding sites are present in the c-myc replicator, but are inaccessible until their exposure is promoted by chromatin acetylation.

Tethered Orc2 binding restored origin activity

Orc2 tethering to a GAL4-binding site has been reported to recruit pre-RC proteins, induce local histone H4 K12 acetylation and promote replication of plasmid or chromosomal origins (26,31). To test whether Orc2 binding was sufficient to induce c-myc replication origin activity, Orc2-GAL4^{DBD} was expressed in the ectopic GAL4-binding site cell lines (Figure 4a). In contrast to previous results using randomly integrated GAL4-binding cassettes (31) or GAL4 plasmids (26), Orc2-GAL4^{DBD} targeting could only restore origin activity at the c-myc-GAL4–binding site, but not at the GAL4-binding site alone (Figure 4b), although the fusion protein bound comparably at these chromosomal sites (Figure 4c and d). We surmise that the random integration used by others (31) may have placed one or more GAL4-binding sites into a permissive environment similar to plasmid chromatin. Consistent with previous demonstrations that tethered ORC could recruit additional pre-RC proteins (26,31), endogenous Mcm7, DUE-B and Cdc45 were bound at the c-myc-GAL4-binding site (Figure 4e–g). However, Mcm7, DUE-B and Cdc45 were not recruited by the Orc2 fusion to the GAL4-binding site alone that lacked adjacent c-myc origin sequences (Figure 4e–g). In contrast, others have reported that tethered ORC could recruit pre-RC proteins to stimulate origin activity in a plasmid (26). Comparison with our results suggests that the plasmid state may be more permissive than the chromosomal environment for both pre-RC formation and origin firing. Moreover, our results support the earlier conclusion that pre-RC assembly at chromosomal origins is separable from replication initiation (10).

Tethered Cdt1 binding restored origin activity

Cdc6 and Cdt1 recruitment to replication origins by ORC are essential to the loading of the MCM replicative helicase (59–66). Consequently, replication-dependent destruction of Cdt1 is one mechanism to prevent reloading of pre-RCs at origins that have already fired (67–72). While pre-RC assembly *per se* does not lead inevitably to origin activity (10), high-level overexpression (>25-30-fold) of Cdt1, or downregulation of its negative regulator geminin, causes re-replication in many cell types (67,68,70,73–78).

GAL4^{DBD}-Cdt1 was expressed in the FRT acceptor cell lines (Figure 5a) and visualized by anti-GAL4 antibody, due to nonspecific reactivity of Cdt1 antibodies. At the present levels of expression, GAL4^{DBD}-Cdt1 did not induce re-replication or genomic instability at its ectopic binding site (Supplementary Figure S3). Nevertheless, the GAL4^{DBD}-Cdt1 protein did induce origin activity at the FRT.myc.5′(930)-GAL4 origin with similar efficiency as the wild-type c-myc replicator (Figure 5b), and bound to the chromosomal GAL4 target with similar occupancy as the E2F1, Orc2 and HBO1 fusion proteins. Intriguingly, tethered GAL4^{DBD}-Cdt1 binding led to the recruitment of Orc2, Mcm7, DUE-B and Cdc45. In conjunction with the restoration of origin activity, these data imply that tethered Cdt1 can directly or indirectly (26,79–83) recruit all essential components of the pre-RC and replisome.

Tethered Mcm7 or Cdc45 failed to restore replication origin activity

Our results, and those of other laboratories (26,31), indicate that chromatin acetylation and early steps in the sequential pathway of ORC, Cdc6, Cdt1, MCM and Cdc45 protein loading at origins could trigger later steps of origin activation. Furthermore, Cdt1 (which loads near the end of pre-RC assembly) could recruit ORC (which is the earliest component in pre-RC assembly). Therefore, we tested whether Mcm7 or Cdc45, which assemble late or after pre-RC assembly, could also recruit proteins that assemble earlier in pre-RC formation and restore origin activity. To this end we expressed GAL4^{DBD} fusions to Mcm7 or Cdc45 in the c-myc-GAL4 cell lines. As shown in Supplementary Figures S4 and S5, tethered binding of Mcm7-GAL4^{DBD} or Cdc45-GAL4^{DBD} failed to recover DNA replication activity at the ectopic c-myc origin. Thus, not all tethered replication proteins can restore origin activity. Furthermore, the tethered binding of Cdc45-GAL4^{DBD} or Mcm7 GAL4^{DBD} did not recruit Orc2, DUE-B Cdc45 (Mcm7-GAL4^{DBD}) or Mcm7 (Cdc45-GAL4^{DBD}). Western blotting demonstrated that the failed recovery of replication activity was not due to poor expression of the fusion proteins. It is formally possible that the GAL4^{DBD} extensions disable Mcm7 and Cdc45, although this seems unlikely inasmuch as these fusion proteins are recruited with site specificity to the DUE of the endogenous c-myc replicator (Supplementary Figure S1).

DUE DNA promotes c-myc origin activity

Deletion of the DUE eliminates origin activity and DUE-B binding at the ectopic c-myc replicator without blocking the binding of Orc2 or Mcm7 (Figure 6) (38). In contrast, AT-rich pentanucleotide repeats $(ATTCT)_{27}$ or $(ATTCT)_{48}$ derived from the ATXN10/SCA10 locus (Gene ID 25814) can functionally replace the c-myc DUE and recruit DUE-B (and Cdc45) to the c-myc replicator (14), but scrambling of the DUE sequence or substitution with another heterologous sequence eliminates c-myc origin activity (40). These results establish a strong correlation between the presence of a DUE, DUE-B binding and origin activity, but do not distinguish between requirements for a DUE sequence versus DUE-B binding. To address this question an additional cell line, HeLa/FRT.myc.GAL4subDUE, was created in which the DUE of the ectopic 2.4 kb c-myc replicator was replaced by the GAL4-binding cassette (pFRT.myc.GAL4subDUE). As shown in Figure 6, substitution of the GAL4-binding cassette for the DUE eliminated binding of DUE-B, Cdc45 and the origin activity of the ectopic c-myc replicator (Figure 6c), without blocking the binding of Orc2 or Mcm7

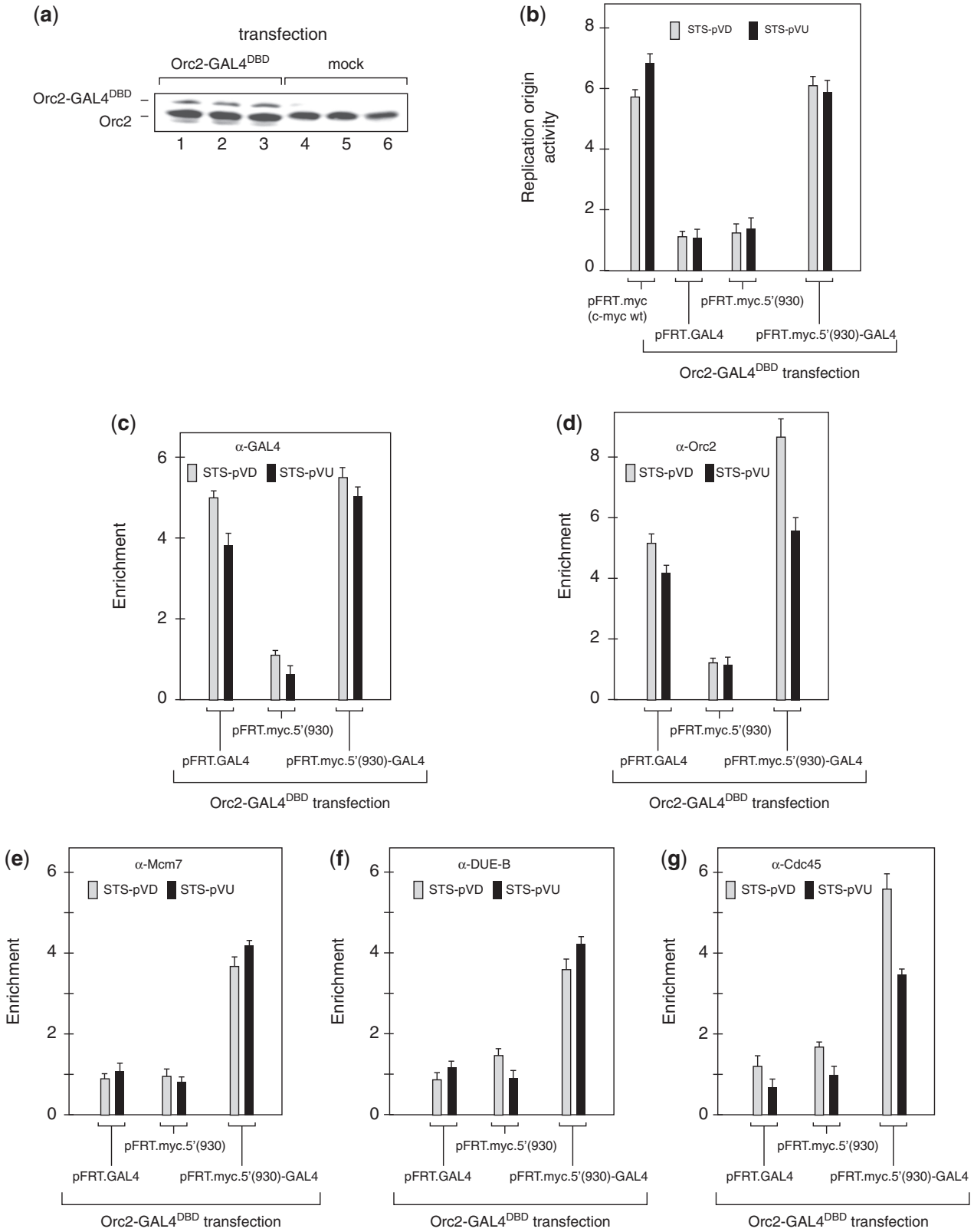

Figure 4. Orc2-GAL4^{DBD} induces origin activity. (**a**) Western blot with anti-Orc2 antibody of extracts from cells containing (lanes 1, 4) FRT.GAL4, (lanes 2, 5) FRT.myc.5′(930), (lanes 3, 6) FRT.myc.5′(930)-GAL4. Lanes 1-3, cells expressing Orc2-GAL4^{DBD}; lanes 4-6, cells transfected with vector only. (**b**) Replication origin activity measured as nascent strand abundance at STS-pVU and STS-pVD in cells containing FRT.myc (WT), or cells expressing Orc2-GAL4^{DBD} and containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4. (**c**-**g**) ChIP using the indicated antibody (α) was quantitated by qPCR at STS-pVU and STS-pVD in cells containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4.

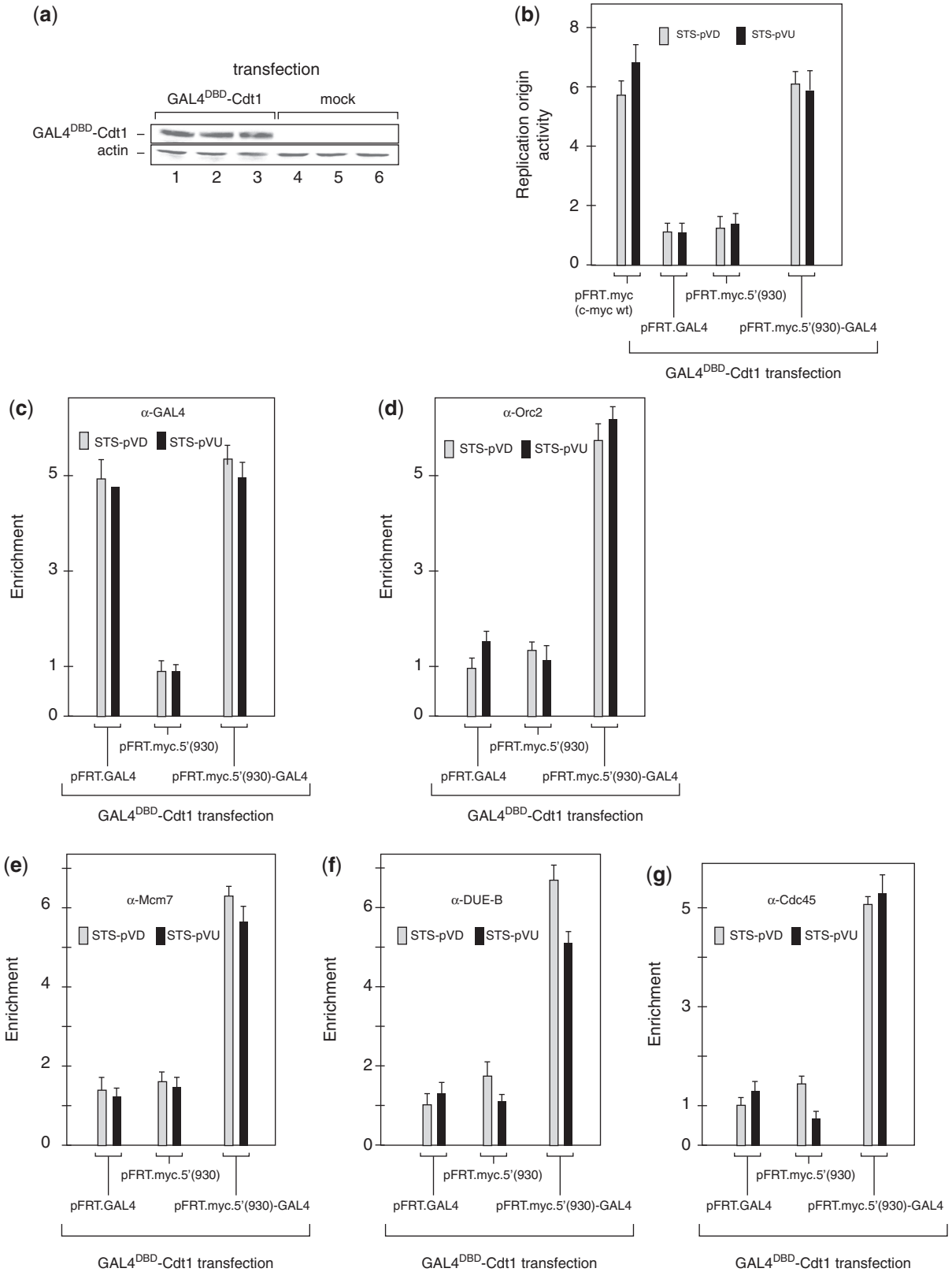

Figure 5. GAL4^{DBD}-Cdt1 induces origin activity. (**a**) Western blot with anti-GAL4 antibody of extracts from cells containing (lanes 1, 4) FRT.GAL4, (lanes 2, 5) FRT.myc.5′(930), (lanes 3, 6) FRT.myc.5′(930)-GAL4. Lanes 1-3, cells expressing GAL4^{DBD}-Cdt1; lanes 4-6, cells transfected with vector only. (**b**) Replication origin activity measured as nascent strand abundance at STS-pVU and STS-pVD in cells containing FRT.myc (WT), or cells expressing GAL4^{DBD}-Cdt1 and containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4. (**c-g**) ChIP using the indicated antibody (α) was quantitated by qPCR at STS-pVU and STS-pVD in cells containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4.

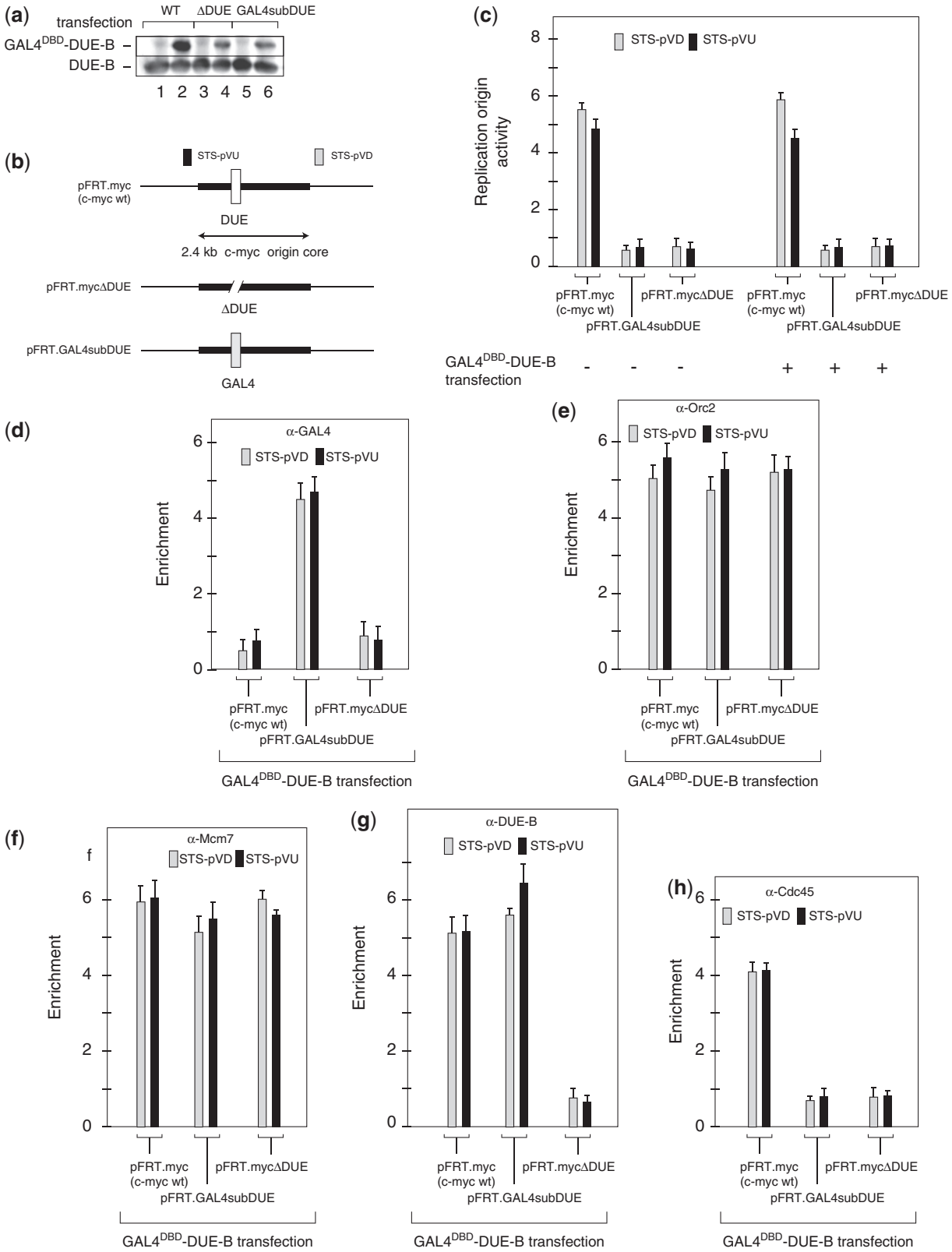

Figure 6. DUE-B does not induce origin activation in the absence of a DUE. (**a**) Western blot with anti-DUE-B antibody of extracts from cells containing (lanes 1, 2) FRT.myc, (lanes 3, 4) FRT.myc.ΔDUE, (lanes 5, 6) FRT.myc.GAL4subDUE. (Lanes 2, 4, 6) cells expressing GAL4^{DBD}-DUE-B; (lanes 1, 3, 5), cells transfected with vector only. (**b**) Maps of cell line constructs used in this figure. (**c**) Replication origin activity measured as nascent strand abundance at STS-pVU and STS-pVD in cells containing FRT.myc (WT), or cells expressing GAL4^{DBD}-DUE-B and containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4. (**d-h**) ChIP using the indicated antibody (α) was quantitated by qPCR at STS-pVU and STS-pVD in cells containing FRT.GAL4, FRT.myc.5′(930), FRT.myc.5′(930)-GAL4.

(Figure 6e and f). These results are consistent with our earlier demonstration that Orc2 and Mcm4 are bound at the dormant ATXN10/SCA10 replication origin, which becomes activated and binds DUE-B, Cdc45 and RPA on $(ATTCT)_n$ expansion (13).

Although the GAL4^{DBD}-DUE-B fusion protein bound selectively to the endogenous c-myc origin DUE (Supplementary Figure S6), tethering of GAL4^{DBD}-DUE-B to the ectopic FRT.myc.GAL4subDUE replicator could not recruit Cdc45 or restore origin activity. Therefore, the DUE sequence itself is necessary for Cdc45 loading and origin activation, but the presence of DUE-B is not sufficient. Taken with data showing that DUE-B is essential for replication in Xenopus egg extracts (13,22), these results suggest that both DUE-B and a DUE are required for ectopic c-myc replicator activity.

DISCUSSION

Chromatin acetylation, Orc2 or Cdt1 recruit pre-RC components in the presence of c-myc origin DNA

Tethering of wild type or mutant GAL4^{DBD} fusion proteins to a GAL4-binding cassette at the ectopic c-myc replicator allows direct assay of their effects on origin activity without targeting endogenous protein expression. As assayed by ChIP, each of the fusion proteins bound to the c-myc origin with comparable affinity and site specificity as their endogenous protein counterparts. This observation argues that the fusion proteins retained normal protein-protein interactions and biological activity. In contrast to previous work of others (31,84), the c-myc replicator reporter constructs and control constructs have been integrated at the same single-copy FRT site in independent clonal HeLa cell lines.

Enhanced histone acetylation occurs at active replication origins (85–88). The present results show that histone H4 acetylation as a result of tethering E2F1 or HBO1 to the FRT.myc.5′(930)-GAL4 replicator correlated with the recruitment of Orc2, DUE-B, Mcm7 and Cdc45. In contrast, decreased histone acetylation by expression of the histone-binding domain (HBD) of the Set8 H4-K20 histone methylase blocked the loading of Mcm5 but not Orc2 at the chr16/axin1 origin (35). Taken together, these data imply that the chr16/axin1 origin is accessible for ORC binding independent of histone acetylation, while ORC binding to the mutant FRT.myc.5′(930)-GAL4 replicator is blocked by the absence of additional stimulatory factors. Importantly, the present results show that H4 acetylation by itself is not sufficient to induce origin activity, and the presence of the 930 bp truncated c-myc origin sequence is also required for origin activity (Figure 2). Possibly, chromatin acetylation promotes nucleosome remodeling and exposure of pre-RC–binding sites in the truncated origin (32,38).

ORC binding to replication origins in budding yeast is dictated by DNA sequence, whereas ORC binding to metazoan replication origins lacks DNA sequence specificity but is enhanced by local negative superhelicity (6,7,89,90). Our results may be reconciled with those of Takeda et al. (26), i.e. that tethered binding of GAL4^{DBD}-Orc2 to a plasmid GAL4-binding sequence conferred replication origin activity in human cells, as plasmid chromatin may offer a permissive environment for subsequent steps in initiation that is not provided by the chromosomal GAL4-binding site in the absence of proximal replicator sequences. Consistent with this view, direct tethering of Orc2 to the FRT.myc.5′(930)-GAL4 replicator enabled the recruitment of all additional factors necessary for replication initiation. The observation that Orc2 tethered to FRT.GAL4 could not recruit Mcm7, DUE-B, Cdc45 or initiate replication shows directly that the 5′ 930 bp of the c-myc replicator plays a role in the assembly of active replication complexes, consistent with previous reports of DNA sequence elements associated with metazoan replication origin activity (91,92).

Similar to the effects of tethering Orc2, targeting of Cdt1 to the inactive FRT.myc.5′(930)-GAL4 replicator restored Orc2, Mcm7, DUE-B and Cdc45 binding, and origin activity. Regulation of intracellular levels of Cdt1 is a key mechanism for preventing re-replication. Although not predicted by the sequential model for loading of ORC, Cdc6 and Cdt1 (65), the initiation of replication on Cdt1 tethering is consistent with previous reports that high overexpression of Cdt1, or downregulation of the Cdt1 inhibitor geminin, induces unscheduled DNA replication (57,58,60,63–68). In contrast, at the level of expression used here, we were unable to detect an abnormal flow cytometry pattern, re-replication or instability at the ectopic c-myc replicator, or checkpoint activation in FRT.myc.5′(930)-GAL4 cells expressing GAL4^{DBD}-Cdt1 (Supplementary Figure S3). We have considered the possibility that contrary to current dogma, Cdt1 binds to ORC or pre-RCs in solution before binding to DNA. However, the inability of tethered GAL4^{DBD}-Cdt1 to recruit Orc2, Mcm7, DUE-B or Cdc45, to the FRT.GAL4-binding site suggests that these proteins interact with Cdt1 after DNA binding, and emphasizes the involvement of the c-myc replicator 5′ sequences in replication protein binding and initiation. GAL4^{DBD}-Cdt1 bound to DNA may interact directly or indirectly with ORC (63,80–83,93,94), or tethered GAL4^{DBD}-Cdt1 may make the replicator more accessible and seed ORC binding by opening the chromatin structure (34).

Mcm7-GAL4^{DBD} and Cdc45-GAL4^{DBD} proteins could not restore origin activity

In contrast to the targeting of E2F1, HBO1, Orc2 or Cdt1, neither the Mcm7-GAL4^{DBD} nor the Cdc45-GAL4^{DBD} fusion could reactivate the c-myc replicator. C-terminal fusion of Mcm7 to a 9 kD TAP tag or to GFP did not interfere with MCM complex formation or Mcm7 function *in vivo* (95). Similarly, a C-terminal fusion of Cdc45 to GFP was functional in yeast (96), and a C-terminal fusion of Cdc45 to amino acids 1-173 or 155-238 of YFP Venus could bind human Mcm2, Mcm7 and Sld5 *in vivo* (97). Although these examples do not formally disprove that the GAL4^{DBD} interferes with the protein binding, DNA binding or biological activity of Mcm7 or Cdc45, taken with the observations that the

Mcm7-GAL4^{DBD} and Cdc45-GAL4^{DBD} fusion proteins were appropriately recruited to the (non-GAL4) endogenous c-myc origin, we do not believe that these proteins are denatured, too large to bind to the origin or that their binding sites are missing from the ectopic origin. Moreover, it is unlikely that the Mcm7-GAL4^{DBD} or Cdc45-GAL4^{DBD} fusion proteins could not restore origin activity simply because the tethered proteins cannot move with the replication fork, as this would not account for their inability to recruit other replication proteins. Alternatively, when the Mcm7 or Cdc45 fusion proteins are tethered to DNA by the GAL4^{DBD}, the binding sites for other replication proteins may be occluded, or Mcm7 and Cdc45 may only bind ORC-dependent complexes preassembled on DNA, but are not themselves able to recruit one or more essential components of the pre-RC (83,93).

The pre-RC forms without a DUE or DUE-B, but both a DUE and DUE-B are required for origin activity

DUE-B is necessary for normal S-phase progress in HeLa cells (22). In Xenopus egg extracts immunodepletion of DUE-B inhibited the binding of Cdc45 and replication initiation, while a dominant negative form of DUE-B blocked replication initiation at a step after MCM loading but before RPA binding (13,22). The relationship between the origin binding of DUE-B, Cdc45 and RPA is strengthened by the *in vivo* observations that pre-RCs are present at an inactive ectopic c-myc origin in which the DUE has been replaced by 8 or 13 copies of the (ATTCT) pentanucleotide derived from the ATXN10/SCA10, origin, and neither DUE-B, Cdc45 or RPA is bound. However, replacement of the ectopic c-myc DUE-By 27 or 48 copies of (ATTCT) reestablished origin binding of DUE-B, Cdc45, RPA and origin activity (14). Similarly, pre-RCs at the wild-type $(ATTCT)_{13}$ ATXN10/SCA10 replication origin are inactive, and DUE-B, Cdc45 or RPA are not bound; however, ATXN10/SCA10 origin activity and binding of DUE-B, Cdc45, RPA occurs in cells from spinocerebellar ataxia type 10 patients containing $(ATTCT)_n$ expansions (14).

Taken together these results show a strong relationship between the presence of a DUE of sufficiently low free energy of unwinding, origin activity and the binding of DUE-B, RPA and Cdc45, but they do not distinguish between the need for an easily unwound DUE versus DUE-B binding for origin activation. To address this issue, GAL4^{DBD} DUE-B was tethered to the GAL4-binding site in place of the ectopic c-myc replicator DUE. Consistent with previous observations that deletion of the DUE did not eliminate pre-RC formation at the c-myc replicator (38), Orc2 and Mcm7 were bound at the FRT.myc.GAL4subDUE replicator in the absence of the DUE or DUE-B, but origin activity and Cdc45 binding were not restored even with tethering of GAL4^{DBD}-DUE-B. These results imply that a DUE sequence is essential for c-myc replicator activity in addition to DUE-B binding. The MCM complex and DUE-B both contain Cdc7 target-binding motifs in superimposable secondary structures, and Cdc7 phosphorylation regulates the MCM/DUE-B interaction in Xenopus egg extracts (Y. Gao, M. Leffak, in preparation). In this context, it appears that Cdc7 phosphorylation may catalyze the release of Cdc45 from DUE-B to the MCM complex coincident with the transition from latent binding of double-stranded DNA to extrusion of one DUE strand from the pre-RC (98). Activation of the CMG (Cdc45-MCM-GINS) helicase leads to origin unwinding, RPA binding and release of DUE-B. It appears that in the absence of a functional DUE, Cdc45 is released from DUE-B by Cdc7 (Y. Gao and M. Leffak, in preparation). Additional experiments are underway to test this hypothesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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