The c-myc DNA Unwinding Element Binding Protein
Modulates the Assembly of DNA Replication Complexes In Vitro

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Running title: Human replication origin DUE binding protein
Summary

The presence of DNA unwinding elements (DUEs) at eukaryotic replicators has raised the question of whether these elements contribute to origin activity by their intrinsic helical instability, as protein binding sites, or both. We used the human c-myc DUE as bait in a yeast one-hybrid screen and identified a DUE-binding protein, designated DUE-B, with a predicted mass of 23.4 kDa. Based on homology to yeast proteins DUE-B was previously classified as an amino acyl tRNA synthetase, however the human protein is approximately 60 amino acids longer than its orthologues in yeast or worms, and is primarily nuclear. In vivo, chromatin-bound DUE-B localizes to the c-myc DUE region. DUE-B levels are constant over the cell cycle although the protein is preferentially phosphorylated in cells arrested early in S phase. Inhibition of DUE-B protein expression slowed HeLa cell cycle progression from G1 to S phase, and induced cell death. DUE-B extracted from HeLa cells or expressed from baculovirus migrates as a dimer during gel filtration, and co-purifies with ATPase activity. In contrast to endogenous DUE-B, baculovirus expressed DUE-B efficiently forms high molecular weight complexes in Xenopus egg or HeLa extracts. In Xenopus extracts baculovirus expressed DUE-B inhibits chromatin replication and RPA loading in the presence of endogenous DUE-B, suggesting that differential covalent modification of these proteins can alter their effect on replication. Recombinant DUE-B expressed in HeLa cells restored replication activity to egg extracts immunodepleted with anti-DUE-B antibody, suggesting that DUE-B plays an important role in replication in vivo.
Introduction

The initiation of DNA replication in eukaryotes relies on the sequential assembly of protein complexes at replicator sequences, controlled by the activities of kinases and phosphatases (1). Genetic and biochemical studies in S. cerevisiae, D. melanogaster and X. laevis suggest that the origin recognition complex (ORC\(^1\)) is a component of the replication initiator that recruits Cdc6, Cdt1 and the minichromosome maintenance (MCM) proteins to origins late in mitosis to form the prereplication complex (pre-RC). Activation of the pre-RC for replication requires the activity of S phase cyclin-dependent kinases plus the Cdc7/Dbf4 kinase, and involves binding of MCM10, Cdc45 and RPA to unwind DNA and load DNA polymerases. A complex containing MCM proteins and Cdc45 may function as a replicative helicase to extend the unwound origin DNA (2,3).

In S. cerevisiae chromosomal replication origins cloned in plasmids display autonomous replicating sequence (ARS) activity, and characteristically comprise a set of modular elements including an ARS consensus sequence (ACS) (4) binding site for ORC (5), a region of helical instability termed a DNA unwinding element (DUE) that contributes to origin activity through template unwinding or binding of pre-RC proteins (6-10) and transcription factor binding sites that can promote the assembly of replication complexes through protein-protein interactions and modification of chromatin structure. In mammalian chromosomes no consensus DNA sequence analogous to the yeast

\(^1\) Abbreviations: 3-AT, 3-aminotriazole; ARS, autonomously replicating sequence; ACS, ARS consensus sequence; DUE, DNA unwinding element; DUE-B, DUE binding protein; ChIP, chromatin immunoprecipitation; MCM, minichromosome maintenance; ORC, origin recognition complex; pre-RC, pre-replication complex.
initiator binding site has been identified. Instead the feature most common to mammalian origins is a region of helical instability (11). Whereas defined sequences derived from the β-globin, lamin B2 and c-myc loci display replicator activity at ectopic loci (12-18), deletion of the 40 kb region encompassing the DHFR ori-β did not eliminate replication initiation in that endogenous location (19), suggesting that ectopic assays reveal the minimal elements essential for replication.

The 2.4 kb upstream region of the human c-myc gene contains multiple transcription factor target binding sites (20). Our laboratory initially reported that replication initiates in this region (21-23), and Vassilev and Johnson were the first to use PCR quantitation of nascent DNA to define the replication initiation zone (24). Mapping of DNA nascent strands by our laboratory (15,18,21-23,25-28) and confirmed by others (24,29-34) showed that replication initiates at multiple sites within this core domain and at flanking sites at the endogenous c-myc chromosomal location (27,28,32). Site-specific chromosomal integration at an ectopic site mediated by the S. cerevisiae FLP recombinase showed that the c-myc origin core satisfies the genetic criteria of a chromosomal replicator, and that a short segment of the c-myc replicator containing the DUE (26,35,36) and three matches to the yeast ACS is essential for replicator activity (15,18).

In eukaryotes, chromosomal replication contrasts with the replication of certain viral genomes (e.g. SV40) in terms of the number of proteins that intervene between the replication initiator and the cellular DNA polymerases. In an attempt to identify additional proteins that might modulate c-myc origin activity we used a yeast one-hybrid assay to isolate proteins that bind to the c-myc DUE/ACS region. We report one such protein,
designated DUE-B, which has a predicted molecular weight of 23.4 kDa and shows strong evolutionary conservation in yeast, mice, frogs and flies. In HeLa cells DUE-B is a constitutively expressed protein found attached to chromatin and in the soluble fraction of lysed cells. During gel exclusion chromatography of HeLa cell nuclear extracts DUE-B migrates at a size of ~46 kDa. Similarly, DUE-B expressed from a baculovirus vector chromatographs with an apparent molecular size of 46 kDa, and copurifies with ATPase activity. In contrast, a significant portion of baculovirus-expressed DUE-B mixed with Xenopus egg or HeLa cell nuclear extracts elutes as a high molecular weight (>250 kDa) species. Chromatin immunoprecipitation assays also show that DUE-B binds at or near the c-myc replicator DUE in a cell cycle dependent manner in vivo.

Consistent with a possible role in replication initiation, DUE-B is preferentially phosphorylated in HeLa cells arrested early in S phase relative to cells blocked at G1 or G2/M. Baculovirus-expressed DUE-B binds saturably to Xenopus sperm chromatin and inhibits its replication in Xenopus egg extracts. In this system DUE-B does not inhibit pre-RC formation, but reduces replication in proportion to its inhibition of RPA binding. In HeLa cells, downregulation of DUE-B protein expression by siRNA is associated with a prolonged G1 phase and the induction of cell death. These data suggest that the c-myc DNA unwinding element binding protein DUE-B plays a role in regulating replication initiation in HeLa cells.
Experimental Procedures

One-hybrid screen

The wild type DUE/ACS region of the c-myc origin (nt 735-832; Genbank accession number X00364) was cloned into the vector pHisi-1 and transformed into S. cerevisiae strain YM4271 (MATa, ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-901, tyr1-501, gal4-Δ512, gal80-Δ538, ade5:hisG). The wild type and mutant DUE/ACS bait sequences are given in Table 1.

The bait sequences were amplified by PCR using primers to allow cloning between the SstI and EcoRI sites of pHisi-1. Yeast transformants were selected for growth on SD/-His medium (Clontech). The DWAW reporter strain was transformed with a HeLa cDNA library cloned in pGAD-GH (Clontech) to produce Gal4AD fusion proteins, and colonies selected for growth at 30°C on his-, leu- medium containing 15 mM 3-aminotriazole (3-AT). Plasmids were isolated from crude yeast lysates and cloned in E. coli according to standard procedures. The identity of yeast strains containing mutant bait sequences was confirmed by PCR amplification and restriction enzyme digestion of the bait sequence, and these strains were transformed with plasmid expressing the Gal4AD-DUE-B fusion protein. Yeast doubling times were calculated by overnight growth in selective medium, dilution to OD600 = 0.1 in selective medium containing 2 mM 3-AT. Doubling times were calculated from the linear portion of growth curves by monitoring the growth at OD600 from 0.3-0.9. Doubling times in the absence of 3-AT were the same for all four reporter strains while yeast transformed with plasmid containing only the Gal4 activation domain did not grow in medium containing 2 mM 3-AT. Plasmid expressing the Gal4AD-DUE-B fusion protein also resulted in elevated
expression of a DUE/ACS:pLacZi reporter integrated at the Ura3 locus in YM4271 (not shown).

**RNA and protein analysis**

RNA was isolated using Trizol, and DUE-B RNA was visualized by Northern blotting of total RNA electrophoresed on denaturing formaldehyde/agarose gels using a DUE-B cDNA probe labeled with \( \alpha^{32P} \)-dCTP by random primer extension.

A cDNA encoding the DUE-B protein with a C-terminal (his)\(_6\) tag was cloned by PCR, inserted into the bacterial expression vector pTRCHis2B and expressed in E. coli induced by 1.0 mM IPTG. The protein was isolated using Ni\(^{2+}\)-NTA agarose under nondenaturing conditions. Polyclonal antibody to DUE-B was produced commercially in rabbits (Cocalico Biologicals Inc.) by injection of DUE-B expressed in E. coli. HeLa cells were synchronized by overnight incubation with 1 ug/ml aphidicolin, 2 mM hydroxyurea, 10 uM purvalanol A, or 100 ng/ml or 400 ng/ml nocodazole. HeLa cells were lysed using NE-PER buffers (Pierce) to yield nuclear and soluble fractions. Western blotting was performed on proteins resolved on 13% SDS-PAGE gels transferred to Immobilon membranes by standard procedures. For phosphate labeling of DUE-B, cells were grown overnight in phosphate-free medium, and labeled for 4 hr with 30 \( \mu \)Ci/ml \( \gamma^{32P} \)-ATP. For expression in insect cells using the MaxBac kit (Invitrogen) DUE-B cDNA was cloned into the pBlueBac4.5 vector and cotransformed with Bac-N-Blue AcMNPV DNA into Sf9 cells according to the manufacturer's directions. Baculovirus-expressed recombinant DUE-B or control Sf9 cell lysates were chromatographed on Ni\(^{2+}\)-NTA resin under nondenaturing conditions (Qiagen).
Chromatography of recombinant DUE-B (200-1000 ng in 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl$_2$) or cell extracts from HeLa cells or Xenopus eggs (2 mg in 50 mM HEPES, pH7.5, 5 mM MgCl$_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10% glycerol, 0.01% Tween 20, 400 mM NaCl) was performed on a one meter Sephacryl S-200 column. HeLa extracts were prepared according to Vashee et al. (37), except that nuclei were extracted with 500 mM NaCl and clarified by microcentrifugation and 0.22 um filtration. Protein elution was monitored by immunoblot or ELISA using antibodies to DUE-B or the (his)$_6$ tag. ATPase assays (25 ul) contained 200 mM Hepes, pH 7.5, 0.01% NP-40, 500 mM NaCl, 10 mM MgCl$_2$, 10 mM DTT, 0.5 mg/ml BSA, 200 uM ATP, 2.7 uM $\gamma$-$^{32}$P-ATP. ATPase activity was monitored by thin layer chromatography on PEI cellulose (38).

EMSA:

A 123 bp probe containing the c-myc DUE/ARS was labeled by PCR in the presence of $\alpha$-$^{32}$P-dCTP. The sequence of the probe is

GAAGGAATTCTAGGAGAATGTTTTTTTTTTTTTCTGCGTGGAATAACAC
AAAATAAAAAATCCCGAGGGAAATATACATTATATATTTAATATAGATCATTTTCAGG
AGCTCGAGAAACA. Additional probes prepared with substitution mutations correspond to the sequences described in the one-hybrid section of Methods. Recombinant DUE-B was purified from baculovirus-infected Sf9 insect cells. Binding reactions (10 mM Tris pH=7.5, 4% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT and 1 mM MgCl$_2$) contained 25 fmol (1.0 ng) probe, 6 pmol (0.15 ug) DUE-B, and 250 ng poly (dl-dC)-poly (dl-dC). Samples were incubated at 30° C for 30 minutes and separated by 4% native PAGE at room temperature in 0.5X TBE buffer.
**Immunocytochemistry**

DUE-B cDNA including C-terminal V5 and (his)$_6$ epitope tags was cloned into pcDNA3.1 and transfected into HeLa cells using Lipofectamine 2000 (Gibco BRL). Twenty-four hours post-transfection the cells were fixed, permeabilized, and sequentially incubated with monoclonal antibodies to either the V5 (Zymed) or (his)$_6$ (Invitrogen, C-terminal specific) epitopes and then FITC conjugated goat anti-mouse IgG (Sigma). Cells were counterstained with Hoechst 33258.

**Nuclease digestion**

HeLa cells were washed with cold phosphate buffered saline and lysed in RSB/NP40 (0.5% NP-40, 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$) at $4^\circ$ C. Nuclei were resuspended in RSB in the presence of 1 mM Ca$^{2+}$ and MNase (5 units/A$_{260}$), or RNase A (5 units/A$_{260}$). Nuclear supernatants and pellets were separated by microcentrifugation and assayed by Western blotting.

**ELISA**

Polystyrene 96 well plates were coated with 100 ng of Sf9 insect cell expressed DUE-B per well or 100 ul of alternate column chromatography fractions. The primary antibodies were used at dilutions of 1:1000 DUE-B polyclonal antiserum or 1:2000 anti-(his)$_6$ antibody and secondary HRP conjugates were used at 1:10,000 dilutions. Signals were assayed by adding 100 ul of OPD/H$_2$O$_2$ substrate (Sigma). Reactions were quenched by the addition of 25 ul 3N HCl and absorbance was read at 490 nm. In
sperm chromatin binding assays, wells were coated with ~23,000 demembranated sperm.

**Chromatin immunoprecipitation**

ChIP was carried out according to the protocol of (39) with the following modifications. Crosslinked chromatin was resuspended in TE buffer and sonicated (Branson sonifier cell disrupter 200, 50% duty cycle, 10 sec pulses, 7 pulses with 1 min intervals). The chromatin was digested with 0.1U micrococcal nuclease (Sigma) per 100 µg of nucleoprotein at 37°C for 5 min to yield fragments <500 bp.

250 µg of nucleoprotein preparation was used for each chromatin immunoprecipitation assay. The nucleoprotein was diluted with 11x NET (550 mM Tris-Cl, pH 7.4, 1.65 M NaCl, 5.5 mM EDTA, 5.5% NP40) to a final concentration of 1X NET. 15 µg of polyclonal DUE-B antiserum or an equivalent amount of normal rabbit antiserum (Upstate Biotech) was used for immunoprecipitation. The antibodies were allowed to bind the chromatin complex for 2 hr at room temperature. Protein A agarose beads supplemented with salmon sperm DNA (Upstate Biotech) were incubated with the antibody complex for another 2 hr at room temperature. The antibody complex washing and purification of co-precipitated DNA was carried out according to (40). Real time PCR was performed as described (15) 1/60 and 1/120 aliquot of immunoprecipitated or input DNA respectively was used for each real time PCR. Primer sequences used for real time PCR are shown in Table 2.
RNAi

Cells were plated in 6 well dishes at 3-5x10⁴/well in α-MEM/5 mM glutamine with 10% fetal bovine serum (GIBCO). siRNA (2 ug in 100 ul Opti-MEM) was mixed with 60 ul of 1:4 Oligofectamine (Gibco):Opti-MEM. Half of the mixture was added to each of two wells and cells were incubated for 48 hr, retransfected and incubated for an additional 48 hr before harvesting. The targets for siRNA are the DUE-B sequences AAGCACUGGUCGAAGAGUGUG (siRNA1) and AAACAGUACGAGAUUCUGUGU (siRNA2). A UU dinucleotide was located at the 5’ end of each siRNA.

Replication in Xenopus egg extracts

Egg high speed cytosol extracts, membranes, sperm chromatin and NPE were prepared and used in replication assays as described (41,42). Replication reactions (30 ul) contained 150 ng M13 phage DNA or chromatin from 50,000 Xenopus sperm. Sperm chromatin binding assays were performed with 1 ug recombinant DUE-B or an equal volume (1 ul) of control buffer pre-incubated in 15 ul cytosol for 30 minutes before the addition of 400,000 demembranated sperm to a final volume of 20 ul. The reaction was incubated for 30 minutes at room temperature and centrifuged twice through a 0.75 M sucrose cushion in a Beckman E microfuge. The sucrose cushions and wash buffers contained 0.5% Triton X-100. The final pellet was resuspended in TE and a 20% aliquot was quantitated by Sybr Green (Molecular Probes) fluorometry using a sperm chromatin standard curve. The remaining solubilized pellet was assayed by Western blot using a monoclonal antibody to MCM7 (Sigma) or RPA70 (NeoMarkers) and SuperSignal West.
Pico chemiluminescent substrate (Pierce). Frogs were used according to guidelines of the WSU Laboratory Animal Care and Use Committee.

To deplete XI DUE-B from egg extracts 0.2 vol preimmune or DUE-B antisera-coupled Protein G agarose beads was incubated with extract for 25 minutes at 4° C three times, as described previously (Walter and Newport, 1999). HeLa DUE-B was purified by nickel affinity chromatography (Ni-NTA agarose, Qiagen) from whole cell lysates (prepared with M-PER (Pierce) and protease inhibitor cocktail (Sigma)) from a clonal HeLa cell line stably overexpressing the DUE-B cDNA with a 6XHis tag.

**Results**

**One-hybrid screen**

Deletion or substitution of the c-myc DUE/ACS region strongly suppresses chromosomal replicator activity (15). To identify proteins that bind to the DUE/ACS, this region was cloned upstream of a His3 reporter gene in the S. cerevisiae His' strain YM4271 (Figure 1A). This strain was transfected with a Gal4 activation domain/HeLa cDNA fusion protein expression library and selection was applied for elevated expression of His3. One cDNA promoted rapid colony growth under selective conditions in strains containing the wild type DUE/ACS sequence but not in otherwise isogenic strains with mutations in the DUE (Figure 1A, 1B). These results imply that the expressed protein interacted specifically with the wild type DUE reporter. The expressed protein was named DUE-B to designate it as a DNA unwinding element binding protein. A reporter strain containing a DUE/ACS bait sequence with mutations in the ACS that
prevent S. cerevisiae ORC binding (4) produced a slight further enhancement of cell growth (Figure 1A, 1B), suggesting that yeast proteins interacting with the c-myc ACS may block access of the Gal4AD:DUE-B fusion protein or other transcription factors to the reporter.

**Evolutionary conservation of DUE-B**

Sequencing of the 1198 bp cDNA revealed a DUE-B open reading frame of 209 amino acids (Table 3) that had been provisionally annotated as a human histidyl amino acyl tRNA synthetase (HARS2, Genbank accession NM080820) (43). However, this designation has not been functionally validated. Human DUE-B has notable similarity to proteins of comparable size in M. musculus (98% similarity), X. laevis (89% similarity), and D. melanogaster (69% similarity, not shown). Proteins of ~60 fewer carboxy terminal amino acids are found in S. cerevisiae (46% similarity) and other organisms (C. elegans, 72% similarity; S. pombe, 45% similarity; and E. coli, 41% similarity, not shown). The 148 amino acid protein in S. cerevisiae with homology to human DUE-B has been identified as a D-tyrosyl-tRNA\(^{tyr}\) deacylase and is nonessential (43).

DUE-B amino acids 29-147 are >90% similar to a domain of unknown function (DUF154) evolutionarily conserved in bacteria, yeast, and mammals (Figure 1C). The C-terminal 70 amino acids of DUE-B show ~50% similarity to nuclear proteins found in humans, flies and mice. The C-terminal 60 amino acid extension of the human protein not found in the worm or yeast enzymes is conserved in the frog (70% identical), mouse (93% identical) and fly (50% similar) proteins. DUE-B cDNA spans seven exons on human chromosome 20, with the proposed initiator methionine located in exon 2.
Northern blot analysis revealed a single species of ~1.4 kb DUE-B mRNA (Figure 2A), that is sufficient to encode a protein of 209 amino acids.

**DUE-B expression in HeLa cells and Xenopus eggs**

Rabbit polyclonal antiserum was raised against the protein expressed in E. coli from the open reading frame of DUE-B fused to a (his)$_6$ tag (Figure 2B, lane 1). This antiserum reacts with a major band migrating at the predicted size of the DUE-B protein (23.4 kDa) (Figure 2B, lane 2). The 23.4 kDa band is immunoprecipitated with the anti-DUE-B serum but not by preimmune serum (not shown). Crossreacting proteins of like size have been detected in human WS1 and HCT116 cells, and in D. melanogaster SN2 cells. Additionally, Xenopus laevis expressed sequences predict a 23.2 kDa DUE-B homologue of 207 amino acids that is 81% similar to human DUE-B. The antiserum produced against human DUE-B was able to detect a band at this molecular weight in Xenopus egg cytosol (see below).

**DUE-B is bound to HeLa chromatin**

To determine the intracellular location of DUE-B, nuclear and soluble lysate fractions were prepared using NE-PER buffers (Pierce) from HeLa cells synchronized by cell cycle inhibitors. DUE-B was detected in both the tightly bound nuclear fraction and in the lysate fraction representing soluble cytoplasmic proteins or proteins loosely associated with nuclei (Figure 2C). Under these fractionation conditions ~ 70% of DUE-B was found in the solubilized fraction, while the histones were quantitatively retained in the nuclei (not shown). However, when cells were lysed in reticulocyte
standard buffer (RSB) containing 0.5% NP-40 ~70% of DUE-B was in the nuclear pellet (Figure 2D, lanes 1, 2). In cells lysed in NE-PER the proportion of DUE-B in nuclear and lysate pools did not vary appreciably between cells arrested in S phase by aphidicolin or hydroxyurea, or in G2/M by nocodazole (Figure 2C). To ascertain whether DUE-B was bound to chromatin, nuclei were isolated from an asynchronous population of HeLa cells and resuspended in RSB (Figure 2D, lanes 1, 2) or RSB containing micrococcal nuclease (MNase, Figure 2D, lanes 3, 4) or ribonuclease A (Figure 2D, lanes 5, 6). DUE-B was quantitatively released from nuclei upon treatment with MNase. The partial release of DUE-B during incubation in RSB with or without RNase was presumably due to the action of endogenous DNases.

To assess the distribution of DUE-B in intact cells the protein tagged with V5 and (his)$_6$ epitopes was expressed in an asynchronous population of HeLa cells. Immunocytochemical analysis using antibody against either the V5 or (his)$_6$ epitope (Figure 2E) revealed that DUE-B is localized primarily to the nucleus, whereas controls using an empty expression vector or omitting the primary antibody against the V5 or (his)$_6$ epitope revealed no nuclear staining (not shown). Taken together with the cell lysis results these data suggest that in vivo DUE-B is present primarily in the nucleus in tightly and loosely bound pools, with the loosely bound portion able to leak into the cytoplasmic lysate during fractionation.

Several proteins associated with yeast replicators undergo CDK phosphorylation prior to origin firing, although the specific function of these reversible phosphorylations is not known. When HeLa cells were treated with cell cycle inhibitors (Figure 3A) and labeled with $\gamma$-$^{32}$P-ATP DUE-B was seen to be phosphorylated in cells arrested in early
S phase by aphidicolin, but showed lower levels of phosphorylation in cells arrested in G1 by mimosine, or in cells arrested in G2/M by the trisubstituted purine purvalanol under conditions which selectively inhibit CDK1/2 (44), and an increased level of phosphorylation in cells treated with the protein phosphatase 2A inhibitor okadaic acid (Figure 3B, C). These results are consistent with the direct or indirect CDK-catalyzed phosphorylation of DUE-B upon entry into S phase and dephosphorylation by PP2A, both of which have been implicated in origin firing (45).

Gel filtration of recombinant and endogenous DUE-B

The DUE-B protein expressed in bacteria elutes from a Sephacryl S-200 column at a position consistent with its predicted monomeric molecular weight of 23.4 kDa (Figure 4A). A (his)$_6$ tagged version of DUE-B was also expressed from a baculovirus vector in Sf9 cells and purified to apparent homogeneity using Ni$^{2+}$-NTA resin (Figure 2B, lane 3). Whereas bacterial expressed DUE-B eluted as a monomer, DUE-B purified from Sf9 cells eluted as an apparent dimer of ~46 kDa (Figure 4A). Thus, expression in eukaryotic cells may enhance DUE-B dimerization by posttranslational modification; interestingly, the related yeast tRNA deacylases are homodimeric enzymes. To determine whether DUE-B exists in a monomeric or multimeric state in vivo, HeLa nuclear extracts were chromatographed. Endogenous DUE-B eluted at a position corresponding to a size of ~46 kDa, with a small percentage of the protein eluting at a higher apparent molecular weight (>250 kDa) (Figure 4B). However, when DUE-B expressed in Sf9 cells was mixed with HeLa nuclear extract roughly three-quarters of the recombinant DUE-B eluted as a dimer, while about one-quarter of the exogenous
protein eluted in a high molecular weight complex near the column void volume (Figure 4C). The complex has a size of ~250-350 kDa on Sepharose 4B chromatography, and is not eliminated by nuclease treatment or by passing the mixture through a 0.22 um filter. The difference in behavior of the endogenous and exogenous proteins suggest that DUE-B is subject to posttranslational modification that affects its ability to interact with other proteins.

In contrast to extracts from an asynchronous population of cultured cells, Xenopus egg extracts offer a highly concentrated pool of proteins poised for rapid and efficient DNA replication. When baculovirus-expressed DUE-B was added to an egg high speed cytosol extract (41) virtually all of the recombinant DUE-B migrated as a >250 kDa complex (Figure 4D). The antibody raised against human DUE-B also detected a band in the Xenopus cytosol preparation that chromatographed at ~46 kDa but electrophoresed at ~23 kDa. The 23 kDa band was not visible when Xenopus cytosol immunoblots were probed with preimmune serum from the same rabbit (not shown). Based on the similarity of its chromatographic, electrophoretic and immunoreactive properties to those of human DUE-B, we refer to the ~23 kDa Xenopus protein band as xlDUE-B.

**Baculovirus-expressed DUE-B co-purifies with ATPase activity**

Several potential purine nucleotide binding sites (motif A, P-loop) were detected in the DUE-B primary sequence. Gel filtration chromatography of the purified baculovirus expressed DUE-B confirmed that an ATPase activity co-eluted with the affinity-purified immunoreactive protein, supporting the view that the ATPase activity is intrinsic or
tightly bound to DUE-B (Figure 5A). This pattern of ATP hydrolysis was not seen with control Sf9 cell lysates (not shown). Fractions 29-34 of the baculovirus-expressed DUE-B were mixed with a 1000-fold molar excess of ATP and the hydrolyzed product quantitated by thin layer chromatography (Figure 5B). At the lowest DUE-B concentration the initial kinetics were linear with a cleavage rate of ~0.35 pmol of ATP hydrolyzed min⁻¹ pmol⁻¹ DUE-B. In comparison, under similar conditions of substrate excess in the absence of DNA, purified yeast ORC is reported to hydrolyze ~0.04 pmol ATP min⁻¹ pmol⁻¹ ORC (46).

Selectivity of DUE-B binding in the presence of HeLa nuclear extract

An electrophoretic mobility shift assay was used to test the specificity of DUE-B binding to the c-myc DUE/ACS origin fragment in vitro. When the baculovirus expressed DUE-B was added to a radiolabeled c-myc DUE/ACS probe DUE-B produced a strongly retarded protein-DNA complex (Figure 6A, lanes 2-4) that was effectively competed by poly (dI-dC)•poly (dI-dC), suggesting that the isolated protein can bind polynucleotides nonspecifically. In the presence of poly (dI-dC)•poly (dI-dC) the baculovirus expressed DUE-B (Figure 6B, lane 1) did not produce a retarded band, while the HeLa extract produced a retarded band of the labeled wild type c-myc DUE/ACS probe (band ‘a’, Figure 6B, lane 3). When recombinant DUE-B was added in the presence of a HeLa nuclear extract, band ‘a’ was decreased in favor of a new band ‘b’, that was resistant to the nonspecific competitor (Figure 6B, lane 2). This result suggests that DUE-B is modified by, or forms a complex with, proteins in the HeLa nuclear extract to overcome the nonspecific competition for binding to the DUE/ACS probe. The retarded band ‘b’
was reactive with the DUE-B antiserum after Western transfer and was supershifted by (his)₆ antibody (not shown).

To test the specificity of this interaction the wild type c-myc DUE/ACS probe (DWAW), recombinant DUE-B, HeLa nuclear extract and poly (dl-dC)•poly (dl-dC) were incubated with a 32- or 64-fold molar excess of unlabeled wild type or mutant versions of the DUE/ACS probe. The unlabeled wild type competitor effectively displaced the DWAW probe (Figure 6B, lanes 4, 5), while the mutant competitors (DMAW: lanes 6, 7; DWAM, lanes 8, 9; and DMAM, lanes 10, 11) were less effective at competing for the wild type DUE/ACS probe. Since DUE-B appeared to bind to the DWAM sequence in the one-hybrid assay, it was expected that the DWAM sequence would compete efficiently in the gel retardation. That it did not may reflect differences in the proteins bound in the in vivo and in vitro assays. Nonetheless, the electrophoretic results suggest that recombinant DUE-B can bind the DUE/ACS region of the c-myc origin with apparent selectivity in the presence of other HeLa nuclear protein(s).

**DUE-B binds near the c-myc DUE in vivo**

Formaldehyde crosslinked chromatin was isolated from HeLa cells growing exponentially or arrested in the G1 phase of the cell cycle, and immunoprecipitated with DUE-B antibody. The abundance of c-myc sequences in the immunoprecipitated chromatin was quantitated by real time PCR (Figure 7). In asynchronously growing cells and in cells arrested in G1 by mimosine, the DUE-B immunoprecipitate showed a significant enrichment for sequences near the c-myc replicator DUE relative to the input chromatin or sequences immunoprecipitated by preimmune serum (Figure 7A, B). The
enrichment for DUE sequences in the DUE-B immunoprecipitates was quantitatively comparable to that seen for MCM4/PRKDC and TOP1 origin sequences in ORC and MCM chromatin immunoprecipitates (47-49). In work to be presented elsewhere, similar enrichments are observed for ORC1, ORC2, MCM3, MCM7 and Cdc6 proteins at the c-myc replicator (M. Ghosh et al., in preparation). The temporal and spatial similarities in the binding of DUE-B and the MCM complex, as exemplified by MCM4, are especially striking (Figure 7C, 7D), possibly reflecting a functional relationship between the DNA unwinding element binding protein and the helicase.

**DUE-B downregulation inhibits cell cycle progress**

In *S. cerevisiae*, disruption of the DUE-B orthologue is not lethal (43). To test whether DUE-B function is essential for cell cycle progression, HeLa cells were transfected twice with siRNA directed against DUE-B mRNA (siRNA1); once at t=0 hr and again at t=48 hr, and examined at t=96 hr without intervening trypsinization (Methods). DUE-B siRNA1 reduced DUE-B levels by >95% but not the levels of the unrelated protein Ku80 (Figure 8A). A scrambled siRNA of similar composition did not reduce DUE-B or Ku80 levels. In four independent experiments, compared to cultures transfected with scrambled siRNA, cultures transfected with DUE-B siRNA1 showed a 3.5- to 8.5-fold increase of cells with sub-G1 DNA content (Figure 8B), a decrease in population doubling (2.5-fold vs. 4-fold) and an increase in cell death (31.5% vs. 9.6%) measured by Trypan Blue exclusion. Transfection of another RNA, DUE-B siRNA2, decreased DUE-B levels more slowly and less completely. DUE-B siRNA2 induced a
smaller increase in cell death (12.5%) and a smaller increase (3- to 5-fold) in the population of sub-G1 cells (not shown).

Cells that had been treated with siRNA1 were trypsinized, synchronized with nocodazole (Figure 8C) and released from the G2/M phase arrest for 12 hr. Relative to cultures treated with scrambled siRNA, cultures treated with DUE-B siRNA1 showed an increase in the population of G1 phase cells (25% vs. 18% control) and a decrease in the fraction of S phase cells (38% vs. 47% control) (Figure 8D). This effect was even more apparent when cells were synchronized in G1 phase with mimosine (Figure 8E) and released (Figure 8F). Here, DUE-B siRNA caused a 50% decrease in the S phase cell population (31% vs. 64%). Taken together, these results suggest that inhibition of DUE-B expression delays entry into S phase and promotes cell death.

**DUE-B controls replication in vitro**

To determine whether DUE-B has a direct effect on replication, Xenopus egg extracts were used. These extracts assemble sperm chromatin into nuclei that undergo a complete round of semiconservative replication, dependent on the ordered assembly of pre-RC components, Cdk2, Cdc7, Cdc45, RPA and DNA polymerase (41,42,50). The Xenopus egg high speed cytosol mimics the in vivo conditions for pre-RC formation, and the subsequent addition of an egg membrane fraction or nucleoplasmic extract (NPE) concentrates factors that promote activation of the pre-RC and replication (41). As shown by gel filtration chromatography (Figure 4) exogenous DUE-B, but not endogenous xIDUE-B, is modified in egg high speed cytosol to form a high molecular weight complex. In an ELISA assay, baculovirus-expressed DUE-B bound saturably to
demembranated sperm chromatin, and the presence of egg extract increased the amount of DUE-B required for 50% sperm chromatin binding approximately 10-fold, from <0.02 ug to 0.2 ug (Figure 9A). In the absence of exogenous DUE-B, binding of the endogenous xIDUE-B was not detected when Xenopus cytosol was incubated with sperm chromatin (not shown).

When DUE-B expressed in bacteria or baculovirus infected Sf9 cells was added to cytosol at roughly the same concentration as endogenous DUE-B (10 ng/ul) simultaneously with sperm chromatin and membranes, the addition of recombinant DUE-B had no apparent effect on DNA replication (Figure 9B, C). However, when recombinant DUE-B was allowed to incubate for 30 minutes with cytosol and sperm chromatin before the addition of membranes, there was a dramatic inhibition of chromatin replication by the Sf9 cell expressed DUE-B and a lesser inhibition of replication by the bacterially expressed DUE-B (Figure 9B, D). By immunoblot assays, both the recombinant and endogenous forms of DUE-B were found to be stable in the Xenopus cytosol and after the addition of membranes (not shown). Baculovirus-expressed DUE-B preincubated with cytosol and sperm chromatin was able to inhibit DNA replication in a dose dependent manner (Figure 9E). By contrast, preincubation of baculovirus-expressed DUE-B with chromatin did not inhibit the formation of sperm pseudo-nuclei (not shown), nor did it inhibit the replication of single stranded M13 DNA, which occurs independently of pre-RC formation (51) (Figure 9F). These observations imply that the inhibitory effect of rDUE-B on replication is not due to a gross perturbation of the egg extract system.
That single stranded DNA replicated normally in the presence of Sf9 expressed rDUE-B suggested that DUE-B is not a general DNA polymerase inhibitor. To determine whether the addition of recombinant DUE-B inhibited replication before or after pre-RC formation the soluble egg cytosol/NPE system was used in a sperm chromatin binding assay. MCM7 and RPA binding were assessed as markers of pre-RC and initiation complex formation, respectively (42,50). DUE-B was preincubated with egg cytosol and sperm chromatin prior to the addition of NPE. Sperm chromatin was separated from the cytosol by sucrose gradient centrifugation in the presence of Triton X-100. The results show a dramatic reduction of RPA binding but no significant decrease in MCM7 loading on sperm chromatin (Figure 9G). To quantitate this effect reactions were performed in triplicate and the results normalized against the amount of sperm chromatin pelleted (Figure 9H). DUE-B did not inhibit MCM7 loading but decreased RPA loading and replication by approximately 80%. The stability of MCM7 binding despite the inhibition of RPA binding and replication argue for the selectivity of DUE-B action on replication initiation in the Xenopus egg extract system.

The inhibitory effect of baculovirus expressed rDUE-B on replication was counterintuitive, however since recombinant and endogenous DUE-B behaved differently upon gel filtration, we decided also to test for a role of endogenous DUE-B in replication. Egg extracts were immunodepleted of xIDUE-B by greater than 95% (Figure 10A) and sperm chromatin replication was measured. In these immunodepleted extracts replication was inhibited by more than 85% (Fig. 10B, C). To determine whether replication could be rescued by DUE-B that had not been expressed in insect cells, HeLa cells were constructed to express hexahistidine tagged rDUE-B. The co-isolation
of rDUE-B and endogenous DUE-B from HeLa cells on nickel affinity chromatography indicates that these proteins fold normally to heterodimerize in vivo. Addition of the HeLa-expressed rDUE-B fraction quantitatively restored replication when added at a four-fold molar excess over the level of endogenous xlDUE-B in undepleted extracts (Figure 10B, C), whereas HeLa cell lysate or Sf9 cell expressed rDUE-B similarly eluted from Ni-NTA did not (data not shown). The observation that removal of DUE-B by immunoprecipitation inhibits replication, and replacement of DUE-B purified by another method, i.e. affinity chromatography, restores replication strongly indicates that DUE-B is involved in the initiation of DNA replication.

Discussion

A previously uncharacterized protein, which we term DUE-B, has been identified based on its selective affinity for the DNA unwinding element of the human c-myc replicator in a yeast one-hybrid screen. In addition to its interaction with an essential element of the c-myc replicator, DUE-B coordinately inhibited sperm chromatin replication and RPA binding in the Xenopus egg extract system, and downregulation of DUE-B slowed entry into S phase and decreased the proliferation of HeLa cells in culture. These observations suggest that DUE-B may play a role in modulating DNA replication in vivo.

Sequencing and translation of the DUE-B cDNA predicted a 23.4 kDa protein of 209 amino acids. Northern blot analysis revealed a 1.4 kb mRNA, sufficient to encode a protein of this size and Western analysis using antibody raised against recombinant DUE-B detected a protein of the predicted size in HeLa cells. Over its N-terminal 148
amino acids DUE-B shows strong similarity (>45% identical, >65% homologous) to yeast and bacterial homodimeric D-tyrosyl-tRNA<sub>tyr</sub> deacylases. While we have not tested for deacylase activity in DUE-B, the S. cerevisiae enzyme is nonessential and cytoplasmic, while a significant portion of HeLa DUE-B is bound to chromatin, and its depletion is associated with decreased cell proliferation. Thus, the yeast and human enzymes appear to have some dissimilar properties. However, the ATPase activity that co-purifies with baculovirus expressed human DUE-B may be related to the hydrolase activity of the yeast enzymes, based on the observation that the seven motif arrangement of invariant amino acids essential for catalysis in the PPM phosphatases is faithfully reproduced in human DUE-B (52). Consistent with a recent report (53), we found the DUE-B cDNA linked to the cDNA of the FK506 binding protein FKBP25 (54), although distinct cDNA libraries were used in these studies and the genes encoding FKBP25 and DUE-B are located respectively on chromosomes 22 and 20. Within the 1198 bp cDNA isolated in the one-hybrid screen, the DUE-B ORF occupies nt 397-1024. Multiple stop codons are present in all reading frames beyond nt 1024, and the sequence complementary to the FKBP25 cDNA (nt 1044-1545) is not part of the FKBP25 ORF. We currently do not have an explanation for this surprising result.

Immunocytochemical analysis showed that epitope-tagged DUE-B expressed in HeLa cells localized to the nucleus in the absence of an added nuclear localization sequence, consistent with the observation that a fraction of endogenous DUE-B was recovered from isolated HeLa nuclei, and released from the nuclei by high salt extraction (not shown) or nuclease digestion, similar to the reported distribution of other pre-RC proteins (55). DUE-B appears to be present at roughly constant levels
throughout the cell cycle and agents that inhibit replication (e.g. aphidicolin, hydroxyurea) did not affect the levels or cellular distribution of DUE-B. Hence, DUE-B does not appear to redistribute between cellular compartments in response to DNA damage. The observation that DUE-B shows preferential binding to the wild type DUE/ACS sequence in vivo, and in vitro in the presence of HeLa nuclear proteins suggests that DUE-B binding to the DUE/ACS may be indirect or involve other proteins that influence the structure of DNA. Recently, Kinoshita and Johnson reported that MCM4 binds near the DUE region of the c-myc replicator preferentially during G1 phase (56). Our data on the binding of MCM4 are consistent with this observation and allow speculation that DUE-B and the MCM helicase recognize or modulate the structure of the DNA unwinding element.

The idea that the binding of DUE-B to the DUE is affected by the proximal binding of other proteins is consistent with the enhanced expression of the one-hybrid reporter in yeast when the ARS consensus elements flanking the c-myc DUE were mutated. Because the GAL4AD-DUE-B protein activated the His3 reporter as well or better when bound to the DWAM bait than to the wild type sequence in the one-hybrid assay, it was expected that the DWAM sequence would compete efficiently in the gel retardation assays. However, comparison of these two assays is difficult since the reporter system is complex and more directly measures transcription than binding. On the other hand, the HeLa proteins that interact with DUE-B do appear to impart sequence selective binding in the context of a nonchromatinized template. The identity of the DUE-B interacting protein(s) is currently under investigation.
On gel exclusion chromatography DUE-B expressed in bacteria eluted at a position consistent with its predicted monomeric molecular size, while DUE-B expressed from a baculovirus vector eluted as a dimer, suggesting that expression in the eucaryotic cells allows posttranslational modifications that affect DUE-B function. Chromatography of HeLa extracts revealed the ~46 kDa dimeric form of endogenous DUE-B, along with a minor amount of DUE-B protein eluting at higher molecular weight (>250 kDa). Roughly one-fourth of recombinant DUE-B added to nuclear extract from an asynchronous HeLa culture was also converted to a high molecular weight form. By contrast, virtually all of the added recombinant DUE-B added to an equivalent amount of Xenopus egg cytosol was found to elute as a high molecular weight complex, while the putative Xenopus DUE-B homolog eluted at the ~46 kDa dimer position. Correlated with the change in association state, the affinity of exogenous DUE-B for sperm chromatin binding decreased 10-fold in the presence of Xenopus egg cytosol. Thus, the high molecular weight complexes containing DUE-B may bind more weakly to chromatin than the dimeric form. The absence of heteromeric complexes between recombinant and endogenous DUE-B strongly suggests that one or both of these molecules is subject to posttranslational modification that induces or precludes high molecular weight complex formation.

In Xenopus egg extracts incubation with baculovirus-expressed DUE-B before the addition of membranes inhibited sperm chromatin replication in a dose-dependent manner. At the approximate concentration of endogenous xIDUE-B, or about one-fourth the molar concentration of MCMs in these extracts (41), exogenous DUE-B inhibited sperm chromatin replication by more than 50%. This inhibition was selective in that
DUE-B did not inhibit the formation of pseudonuclei, the replication of single stranded DNA or the loading of MCM7. In the soluble cytosol-NPE system the inhibition of sperm chromatin replication was quantitatively correlated with the decreased loading of RPA. This does not appear to be the result of a direct interaction between DUE-B and RPA inasmuch as DUE-B and RPA do not co-immunoprecipitate or interact in pull-down assays (M. Kemp, unpublished), and recombinant DUE-B did not inhibit the replication of single stranded DNA. Therefore, to the extent that MCM7 loading reflects pre-RC formation, DUE-B inhibits sperm chromatin replication at a step following pre-RC formation. Endogenous DUE-B does not form high molecular weight complexes and is permissive or stimulatory for DNA replication, while exogenous DUE-B expressed from baculovirus forms high molecular weight complexes and inhibits replication. It is plausible therefore that variation in covalent modification are responsible for both of these differences. Preincubation with the extract may allow the baculovirus expressed DUE-B to act as a dominant negative mutant by competing for or sequestering essential replication factors yet to be identified.

We suggest that one or more components of the high molecular weight complex formed in Xenopus extracts may be homologous to proteins present in limiting amounts in the asynchronous HeLa nuclear extract, and that endogenous and exogenous DUE-B differ in posttranslational modification states such that only exogenous DUE-B is able to form the high molecular weight complex, suppress RPA loading and inhibit replication. A two-state model has also been proposed to explain the difference in stability of endogenous and exogenous geminin in Xenopus egg extracts (57). Taken with the observation that ablation of DUE-B expression is associated with reduced cell
proliferation, DUE-B may play both positive and negative roles in replication initiation. Several origin binding proteins are substrates for CDK-dependent phosphorylation including Cdc6p, MCM4p, Orc2p, and Orc6p (58-61). The increased phosphorylation of DUE-B in early S phase may reflect the transition between these states.

We do not know whether the high molecular weight complexes formed by endogenous DUE-B in asynchronous HeLa extracts are cell cycle regulated or are the same as those formed by recombinant DUE-B. However, the quantitative difference in the amount of high molecular weight complexes in Xenopus and HeLa extracts points to the cell cycle dependent regulation of one or more proteins that stoichiometrically interact with DUE-B. It is proposed that DUE-B is present in vivo in a modified dimeric form that distinguishes it from exogenously expressed DUE-B, and that prior to the initiation of replication DUE-B is further modified to supply a factor necessary for RPA loading at the pre-RC. In this model, baculovirus-expressed DUE-B may sequester this factor in a high molecular weight complex, inhibiting RPA deposition.

Further evidence of physiological differences between Sf9 expressed and endogenous or HeLa expressed DUE-B come from in vitro replication experiments in which depletion of endogenous xIDUE-B or the addition of baculovirus expressed recombinant DUE-B inhibited replication, whereas a HeLa expressed recombinant DUE-B restored replication to immunodepleted extracts when added in four-fold excess over in vivo levels. These results indicate that endogenous DUE-B is important for DNA replication. Among other possibilities, the need for a molar excess of this fraction could mean that only a portion of DUE-B isolated from asynchronous cells is correctly
modified (e.g. phosphorylated) to stimulate replication, or that only the endogenous HeLa DUE-B that heterodimerizes with the hexahistidine tagged DUE-B is active.

Consistent with a model in which DUE-B plays a role in DNA replication and cell cycle progression, we note preliminary results indicating that DUE-B mRNA levels are elevated by 40%-300% in fifteen of twenty ovarian and colon tumors tested relative to neighboring normal tissue (M. Kemp, unpublished). Work is underway to address several aspects of a model for DUE-B in mammalian replication.

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Figure Legends

Figure 1. One-hybrid identification of DUE-B
A. Schematic of c-myc DUE/ACS bait sequences integrated upstream of the His3 reporter. Open boxes, ACS matches. DWAW, DUE wild type, ACS wild type; DWAM, DUE wild type, ACS mutant; DMAW, DUE mutant, ACS wild type; DMAM, DUE mutant, ACS mutant. Vertical hatches indicate positions of mutations. See Methods for DNA sequences. B. Doubling time of reporter strains containing wild type or mutant bait sequences, transfected with a plasmid expressing the Gal4AD-DUE-B fusion protein. Error bars, SD. C. Schematic of DUE-B. DUF154 is a conserved domain of unknown function. Proteins with similarity (%) to DUE-B are indicated. Numbers and brackets delimit regions of amino acid similarity.

Figure 2. DUE-B expression and localization
A. Northern blot of HeLa RNA probed with DUE-B cDNA. B. Lane 1, immunoblot of Ni⁺-NTA purified V5⁺, (his)₆-tagged DUE-B expressed in E. coli. Lane 2, immunoblot of HeLa whole cell extract. Lane 3, silver stain of recombinant (his)₆-tagged DUE-B expressed from a baculovirus vector, Ni⁺-NTA purified from Sf9 cells. Lanes 1 and 2 were probed with rabbit antiserum against E. coli expressed DUE-B. C. Immunoblot analysis of HeLa nuclear and soluble lysate fractions probed with DUE-B antiserum. Extracts were prepared from an asynchronous culture or cultures arrested with the indicated inhibitors. D. Nuclei were isolated from HeLa cells and resuspended in RSB (lanes 1, 2), or RSB plus MNase (lanes 3, 4), or RSB plus RNase A (lanes 5, 6). Nuclei were incubated at 37°C, pelleted and the total nuclear pellets and soluble supernatants were resolved by SDS-PAGE and analyzed by immunoblotting with DUE-B antiserum.
The variation in mobility of DUE-B in the S and P fractions is due to differing buffer conditions since DUE-B migrates as a single band in total cell lysates under normal conditions (e.g. lane 2, panel B). E. HeLa cells expressing V5, (his)$_6$ epitope-tagged DUE-B were visualized by phase contrast microscopy, Hoechst staining and FITC-conjugated murine IgG against the indicated epitope tag.

**Figure 3: DUE-B Phosphorylation.** A. Flow cytometric analysis of HeLa cell cycle distribution in an asynchronous culture (ASY), or in cultures treated with mimosine (MIM), aphidicolin (APHD), purvalanol (PUR), nocodazole (NOC), or okadaic acid (OKA). B. Pulse radiolabeling (4 hr) of DUE-B with $\gamma^{32}$P-ATP in cells treated as in (A). DUE-B was immunoprecipitated with DUE-B antiserum and analyzed by SDS-PAGE followed by autoradiography. Cells were untreated (asynchronous, lane 1), or treated with mimosine (lane 2), aphidicolin (lane 3), purvalanol (lane 4), nocodazole (lane 5), or okadaic acid (lane 6). C. Autoradiographic signals in panel B were normalized to equivalent amounts of immunoprecipitated DUE-B detected by Western blotting.

**Figure 4. Gel filtration chromatography of DUE-B**

A. A Sephacryl S-200 column was calibrated with the indicated standards and the elution peaks of DUE-B expressed in E. coli, or baculovirus infected Sf9 cells were determined by ELISA. The E. coli generated protein has an apparent molecular weight of ~23 kDa while the insect expressed protein has an apparent molecular weight of ~46 kDa. B. Sephacryl S-200 chromatography of HeLa nuclear extract analyzed by immunoblotting. Standards were monitored by Coomassie staining. I, input sample
 aliquot. Arrowhead indicates elution position of the minor high molecular weight DUE-B component. C. Sephacryl S-200 chromatography of HeLa nuclear extract mixed with DUE-B expressed from recombinant baculovirus (rDUE-B). The mixture was incubated for 30 minutes at room temperature, clarified by microcentrifugation and 0.22 um filtration. Alternate fractions were probed using DUE-B antiserum. The elution pattern was not altered by preincubating the mixture with nuclease. hsDUE-B, endogenous DUE-B. D. Sephacryl 200 chromatography of Xenopus egg high speed cytosol mixed with DUE-B expressed from recombinant baculovirus (rDUE-B). Column conditions were as in C. xlDUE-B, putative Xenopus laevis DUE-B. Alternate fractions were probed using DUE-B antiserum.

Figure 5. Recombinant DUE-B expressed in Sf9 cells purifies with ATPase activity
A. Recombinant DUE-B expressed in Sf9 insect cells was purified by Ni\(^{+}\)-NTA chromatography and rechromatographed on a Bio-Rad SEC-250 gel filtration column. Each fraction was assayed by ELISA using DUE-B antiserum (solid circles), or antibody to the carboxy-terminal (his)\(_6\) epitope (squares). ATPase activity (open circles) was monitored by thin layer chromatography of every second fraction incubated with \(\gamma^{-32}\text{P-ATP}\). \(32\text{P}\)\(_i\) was quantitated by PhosphorImager autoradiography of TLC plates. B. Sf9 expressed recombinant DUE-B was incubated with \(\gamma^{-32}\text{P-ATP}\). Time courses of ATP hydrolysis were measured by thin layer chromatography using two levels of Sf9 expressed recombinant DUE-B. Background activity of an equivalent volume of control Sf9 eluate was less than 50 fmol ATP hydrolyzed in 60 minutes.
Figure 6. Sequence selectivity of DUE-B binding in the presence of HeLa nuclear extract

(A) A 123 bp probe (2 ng, 25 fmol) containing the c-myc DUE/ACS region was mixed with Sf9 expressed DUE-B (1.5, 3, 6 pmol) in the absence of poly (dl-dC) • poly (dl-dC), or with 6 pmol DUE-B in the presence of 250 ng of poly (dl-dC) • poly (dl-dC).

(B) The 123 bp c-myc DUE/ACS probe (25 fmol) was mixed with recombinant DUE-B (6 pmol), HeLa nuclear extract (10 ug) and poly (dl-dC) • poly (dl-dC) (250 ng). Lanes 4-11, increasing amounts of the indicated unlabeled competitor in the presence of labeled wild type (DWAW) origin probe. Lanes 4, 6, 8 and 10 contain a 32-fold molar excess (800 fmol) of the indicated competitor; lanes 5, 7, 9, 11 contain a 64-fold molar excess of the indicated competitor.

Figure 7. DUE-B chromatin immunoprecipitation

Crosslinked chromatin was isolated from HeLa cells in exponential growth (A), arrested in G1 with mimosine (B); or arrested in G2/M with nocodazole (C). Chromatin was immunoprecipitated with DUE-B antibody or control preimmune antiserum, and c-myc replicator sequences were quantitated by real time PCR. The abundance of immunoprecipitated sequences is expressed as the enrichment over input (left axis) or enrichment over preimmune immunoprecipitate (right axis). The data are averages of three or four experiments; in all cases, the standard deviation of the measurements was <14% of the value shown. The map indicates the positions of the primer sets used for quantitative PCR. Filled boxes, c-myc exons; shaded box, fragment showing c-myc replicator activity at an ectopic chromosomal site (15,18); DUE, c-myc DNA unwinding element.

Figure 8. siRNA silencing of DUE-B

A. Immunoblot of whole cell lysates probed with DUE-B antibody after siRNA treatment. Lane 1, untransfected; lane 2, mock transfected; lane 3, scrambled siRNA control transfection; lane 4, DUE-B siRNA1 transfection. Cells were transfected at time t=0 hr.
and t=48 hr, and harvested at t=96 hr. B. Flow cytometry analysis of cells harvested after transfection with scrambled siRNA (filled trace) or DUE-B siRNA1 (unfilled trace). The percentage of cells in each cell cycle fraction is shown at the right. C. Nocodazole synchronization of siRNA transfected cells. D. Cells transfected with scrambled siRNA (filled trace) or DUE-B siRNA1 (unfilled trace) were replated, arrested with nocodazole and analyzed 12 hr after release from arrest. E. Mimosine synchronization of siRNA transfected cells. F. Cells transfected with scrambled siRNA (filled trace) or DUE-B siRNA1 (unfilled trace) were replated, arrested with mimosine and analyzed 7 hr after release from arrest.

**Figure 9. Inhibition of replication in vitro**
A. The wells of a 96 well plate were treated with sperm chromatin or buffer, and blocked with BSA. Baculovirus-expressed DUE-B, or DUE-B preincubated with egg high speed cytosol, was added to the wells and the bound DUE-B was quantitated by ELISA (Methods). B. Recombinant DUE-B expressed in bacteria or in Sf9 cells, or control Sf9 cell lysate (Methods) was mixed with Xenopus egg high speed cytosol and sperm chromatin. Membranes and $\alpha^{32}$P-dCTP were added immediately (left) or after 30 min incubation (right). Aliquots were removed at 0, 30, 60 and 90 min, and analyzed by electrophoresis and autoradiography. The electrophoretic banding pattern of replicated sperm chromatin is as observed previously (41,51,62). C. Quantitation of sperm chromatin replication following simultaneous addition of DUE-B (10 ng/ul) and membranes. D. Quantitation of sperm chromatin replication with 30 minute DUE-B (10 ng/ul) preincubation prior to addition of membranes. E. The inhibition of sperm
chromatin replication is dependent on the dose of baculovirus-expressed DUE-B during preincubation. F. Single stranded DNA (M13 DNA) replication is not inhibited by the addition of baculovirus-expressed DUE-B. G. Immunoblot analysis of protein loading onto sperm chromatin incubated in the presence or absence of baculovirus-expressed DUE-B, high speed cytosol and nucleoplasmic extract. H. Effects of baculovirus-expressed DUE-B on replication and protein loading of sperm chromatin in the soluble high speed cytosol plus NPE system. The results of triplicate experiments are shown. The relative amounts of protein loaded are normalized to the amount of sperm chromatin pelleted, measured fluorometrically (Sybr Green). Error bars, SD.

**Figure 10. DNA replication in immunodepleted Xenopus egg extracts can be complemented by human DUE-B.**

A. DUE-B is efficiently removed from egg extracts by DUE-B antisera. Western blot of mock depleted (lane 1, immunodepletion with preimmune serum) or DUE-B depleted (lane 2, 3, depletion with anti-DUE-B antiserum) extracts. Orc2 is included as a loading control. Replication (90 minutes) in mock (lane 1) or DUE-B depleted extracts without (lane 2) or with (lane 3) added purified HeLa DUE-B. Data are representative of multiple independent experiments. C. Quantitation of replication (90 minutes) in mock (lane 1, n=5) or DUE-B depleted extracts without (lane 2, n=4) or with (lane 3, n=5) added purified HeLa DUE-B. Error bars, SD.
Figure 2

A

B

C

D

E

1.5 kb

kDa

36

25

19

DUE-B

asynchronous

nocodazole (1X)
nocodazole (4X)
aphidicolin
hydroxyurea

soluble

nuclear

phase

Hoechst

antibody

anti-(his)6

anti-(his)6

anti-V5

anti-V5

S

P

RSB

MNase

RNase
Figure 3

A

B

C

Arbitrary units: $^{32}$P-DUE-B

MIM

APHD

PUR

NOC

OKA

1 2 3 4 5 6

A M A P N O K A

S Y M H R D
Figure 4

A

log molecular weight

B

DUE-B

C

rDUE-B

hsDUE-B

D

rDUE-B

xIDUE-B

Ve/Vo

catalase
aldoase
rDUE-B (insect)
BSA
rDUE-B (bacterial)
chymotrypsinogen
cytochrome C

aldoase (198 kDa)
BSA (67 kDa)
chymotryps. cyt. C (28 kDa)
(12 kDa)

aldolase
BSA
Figure 5

A

![Graph A]

B

![Graph B]

- anti-(his)₆
- anti-DUE-B
- ATPase activity

Relative ATPase activity

Fraction number

A₅₆₀

0.25

0

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

Relative ATPase activity

0

1000

2000

3000

0

15

30

45

60

Time (minutes)

192 fmol

48 fmol

fmol ATP hydrolyzed
Figure 7

A. DUE-B, Asynchronous

B. DUE-B, G1

C. MCM4, Asynchronous

D. MCM4, G1

[caption or description of the figure]
Figure 10

A

|   | 1 | 2 | 3 |
|---|---|---|---|
| Orc2 | - | - | - |
| DUE-B-6XHis | - | - | - |
| HeLa DUE-B | - | - | - |
| xIDUE-B | - | - | - |

Depletion: Mock DUE-B

B

C

Replication (% of mock control)
Table 1: Yeast one-hybrid assay bait sequences. Bold, ARS consensus matches; underline, DUE; upper case, wild type nucleotides; lower case, mutated nucleotides.

| Sequence          | Wild type (DWAW)                                                                                       |
|-------------------|----------------------------------------------------------------------------------------------------------|
|                  | 5'ATGAGAAGAATGTTTTTGTTCATGCCGTGGAAATAAACAACAAAAATAAAAATCCCCGAGGGAA                                      |
|                  | TATACATTATATATTAAATAGATCATTTTCAGG                                                                       |

| Sequence          | ACS mutant (DWAM)                                                                                       |
|-------------------|----------------------------------------------------------------------------------------------------------|
|                  | 5'ATGAGAAGAATGTTTTTGTGCcTTCATGCCGTGGAAATAACACAgcgTAAAAAAATCCCCGAGGGAA                                  |
|                  | ATACATTATATATTgtTATAGATCATTTTCAGG                                                                        |

| Sequence          | DUE mutant (DMAW)                                                                                       |
|-------------------|----------------------------------------------------------------------------------------------------------|
|                  | 5'ATGAGAAGAATGTTTTTGTTCATGCCGTGGAAATAACACAAAAATAAAAATCCCCGAGGGAA                                      |
|                  | TATACATTcggcTAATAATAGATCATTTTCAGG                                                                       |

| Sequence          | DUE mutant/ACS mutant (DMAM)                                                                            |
|-------------------|----------------------------------------------------------------------------------------------------------|
|                  | 5'ATGAGAAGAATGTTTTTGTGCcTTCATGCCGTGGAAATAACACAgcgTAAAAAAATCCCCGAGGGAA                                  |
|                  | gcACATTgcATATTgcgcgTAGATCATTTTCAGG                                                                      |

Table 2: ChIP PCR primers.

| 1 F  | 5' GGCTCCTGCGGGAAGG |
| 1 R  | 5' CCTGACCGGTGTCTGATCATTAGA |
| 2 F  | 5' TGCCATTACCGGTTCTCCA |
| 2 R  | 5' TTCAACCGCATAAGAGATGGT |
| 3 F  | 5' GGGAAAGACGCTTTGCAGC |
| 3 R  | 5' TTTGCCGCAAACGC |
| 4 F  | 5' TTCAACAGGTGCTCTGACTC |
| 4 R  | 5' GCGGGACCCGGACTTCTCA |
| 5 F  | 5' CGGGCCATTAATACCCTT |
| 5 R  | 5' AGGGCCCGCGCTTTGA |
| 6 F  | 5' CACTTGGCACTGAACTTACAACA |
| 6 R  | 5' GAATAGCCTCCCGCCTC |
| 7 F  | 5' TTGTGTGCCCCGCTCC |
| 7 R  | 5' TTTCGGTTGGTAGCTAGCTT |
| 8 F  | 5' TGGTCTTCCCCCATACCCTCTCA |
| 8 R  | 5' TGGAGTCTTGGCCAGGCG |
Table 3. The amino acid sequence of human DUE-B used in these experiments is compared to the sequences of the mouse, frog and yeast proteins. Solid black boxes indicate identity; grey shaded boxes indicate similarity, white boxes indicate non-similarity. The alignment was constructed using the following sequences: H. sapiens (this work; Genbank BC045167), M. musculus (Swissprot accession Q9DD18), S. cerevisiae (Swissprot accession Q07648), X. laevis (Genbank accessions AW644650, BQ386724).
The c-myc DNA unwinding element binding protein modulates the assembly of DNA replication complexes in vitro
John M. Casper, Michael G. Kemp, Maloy Ghosh, Gia M. Randall, Andrew Vaillant and Michael Leffak

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