A Dominant-negative Mutant of Human DNA Helicase B Blocks the Onset of Chromosomal DNA Replication*

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A cDNA encoding a human ortholog of mouse DNA helicase B, which may play a role in DNA replication, has been cloned and expressed as a recombinant protein. The predicted human DNA helicase B (HDHB) protein contains conserved helicase motifs (superfamily 1) that are strikingly similar to those of bacterial recD and T4 dda proteins. The HDHB gene is expressed at low levels in liver, spleen, kidney, and brain and at higher levels in testis and thymus. Purified recombinant HDHB hydrolyzed ATP and dATP in the presence of single-stranded DNA, displayed robust 5′-3′ DNA helicase activity, and interacted physically and functionally with DNA polymerase α-prime. HDHB proteins with mutations in the Walker A or B motif lacked ATPase and helicase activity but retained the ability to interact with DNA polymerase α-prime, suggesting that the mutants might be dominant over endogenous HDHB in human cells. When purified HDHB protein was microinjected into the nucleus of cells in early G1, the mutant proteins inhibited DNA synthesis, whereas the wild type protein had no effect. Injection of wild type or mutant protein into cells at G1/S did not prevent DNA synthesis. The results suggest that HDHB function is required for S phase entry.

DNA helicases are an abundant class of DNA metabolic enzymes, surpassing even the DNA polymerases in number and complexity, as well as in their resistance to experimental efforts to eludicate their functions. Although prokaryotic and viral DNA helicases are comparatively well studied, eukaryotic DNA helicases remain poorly understood. The 134 helicase-related genes encoded by Saccharomyces cerevisiae constitute more than 2% of the genome, but physiological functions of few of them are known (1). A better understanding of DNA replication, repair, and recombination pathways and the interplay between cells in eukaryotic cells will depend on elucidation of the DNA helicases involved and their roles in each pathway.

SV40 T antigen, a multifunctional viral protein, has served as a paradigm for a replicative helicase in eukaryotes (2, 3). It assembles on the viral origin of DNA replication, unwinds the parental strands, and directs the assembly of the cellular DNA polymerase α-prime (pol-prim) (4) and replication protein A (RPA) (5) on the DNA, mediating the synthesis of the first RNA primers. A cellular DNA helicase, mouse DNA helicase B, was reported to share with T antigen the capacity to load pol-prim on RPA-coated single-stranded DNA and activate RNA primer synthesis (6, 7). Moreover, in a mutant derivative of F93A mouse mammary carcinoma cells that express a thermolabile mutant of murine DNA helicase B, the onset of DNA replication was blocked at the non-permissive temperature (8), consistent with a possible role of the helicase in initiation of DNA replication. A cDNA encoding mouse DNA helicase B was recently cloned and characterized as a member of helicase superfamily 1 (9), which includes several well studied prokaryotic helicases, e.g. Escherichia coli uvrD/Helicase II, rep, recB(CD), and Bacillus stearothermophilus PcrA (10–12). However, recombinant mouse DNA helicase B expressed from the murine cDNA has not yet been described.

To explore the role of a putative human ortholog of mouse DNA helicase B in human DNA replication, we have cloned a cDNA encoding human DNA helicase B (HDHB), expressed and purified the recombinant polypeptide, and characterized its activity in vitro and after microinjection into human cells. The results demonstrate that HDHB is closely related in sequence to the mouse helicase and is widely expressed in cells and tissues with an active DNA metabolism. The properties of recombinant HDHB confirm and extend those reported for the homolog purified from mouse cells, strongly suggesting a role for HDHB in an aspect of DNA metabolism that depends on pol-prim priming activity.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing of Human DNA Helicase B cDNA—A blast search of the Expressed Sequence Tag (EST) database (National Center for Biotechnology Information) revealed human sequences AA070301 (395 bp), AA256818 (311 bp), and AA256278 (258 bp) with significant homology to the mouse helicase and is widely expressed in cells and tissues with an active DNA metabolism. The properties of recombinant HDHB confirm and extend those reported for the homolog purified from mouse cells, strongly suggesting a role for HDHB in an aspect of DNA metabolism that depends on pol-prim priming activity. 

The abbreviations used are: pol-prim, DNA polymerase α-prime; HDHB, human DNA helicase B; FEN-1, flap endonuclease 1; nt, nucleotide(s); RPA, replication protein A; BSA, bovine serum albumin; DTT, dithiothreitol; ssDNA, single-stranded DNA; ATPγS, adenosine 5′-O-(thiotriphosphate); BrdUrd, bromodeoxyuridine; wt, wild type; RT, reverse transcription.
Expression of HDHB in Human Cells and Tissues—Total RNA (1 to 10 µg) from tissue culture cells or human tissues (Clontech, Palo Alto, CA) or from frozen or fresh-frozen brain tissue from patients with Huntington’s disease was reverse-transcribed into cDNA with Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 10 units of RNasin, 100 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using 100 ng of the primer 5′-AATGAGCTCAAGCAGTG-3′ (Integrated DNA Technologies, Coralville, IA). The reaction was incubated at 25 °C for 10 min, 42 °C for 20 min, and 95 °C for 5 min. Samples of this first-strand cDNA product were first incubated with the PCR primers (reverse, 5′-CATCTATGACTGTCCAGG-3′; forward, 5′-GATATTGGTGTGGTGACA-3′) in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM dNTP, and 0.625 units of Taq polymerase for 3 min at 94 °C. PCR amplification of a 403-bp fragment was carried out for 20 cycles (1 min each at 94 °C, 1 min at 55 °C, 2 min, 72 °C). PCR products were blotted onto nylon membranes (Optitran BA-S85; Schleicher & Schuell, Keene, NH), irradiated for 5 min under UV light, and then hybridized with a radio-labeled DNA probe (nucleotides 504–1948 of the HDHB cDNA open reading frame). Hybridization signals were quantified by phosphorimaging. To compare signals from different RNA samples, total RNA (2.5 or 5 µg) was electrophoresed in 1% agarose gels and stained with ethidium bromide, and the rRNA bands were quantified by phosphorimaging using Image Store 7500 (UVP Inc., Upland, CA).

Expression and Purification of Recombinant Human DNA Helicase B—An XbaI/NcoI fragment containing the tagged HDHB cDNA was transferred from the pET-28a (+) vector into a baculovirus transfer vector. For in vitro expression, the recombinant vector and Baculogold DNA (Pharmingen, San Diego, CA) were co-transfected into Sf9 insect cells using DOTAP transfection reagent (Roche Molecular Biochemicals). Sf9 cells were grown in Grace’s insect medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone, Logan, UT). Preparation of recombinant baculovirus and infection of the cells were performed according to published protocols (14). Point mutations in the Walker A (K491A) and Walker B (E95Q) motifs of HDHB were generated in the cDNA insert in pET28 by QuiKchange mutagenesis (Stratagene) according to the manufacturer’s protocol and confirmed by DNA sequencing. Mutagenic primers (Integrated DNA Technologies) were used to generate Walker A and Walker B mutants. The mutant sequences were transferred into pVL1393/HDHB to replace the wild-type sequence. The entire DNA sequence was determined by the chain termination method (22) using a T7 Sequenase DNA sequencing kit (Amersham Biosciences). The DNA sequence data were deposited in the GenBankTM/EMBL database under GenBank accession number AF319995.

DNA-dependent ATPase Assay—The standard reaction mixture (10 µl) contained 20 mM Tris-HCl (pH 7.5), 0.1 µg/ml BSA, 0.5 mM DTT, 10 mM MgCl2, 50 µM [γ-32P]ATP (1 Ci/mmol) (Amersham Biosciences), varying amounts of DNA and RNA, and protein to be tested. Samples were incubated for 15 min at 37 °C, and reactions were terminated with 10 µl of ice-cold 50 mM EDTA. 1 µl of reaction mixture was spotted on a polyethyleneimine-cellulose thin layer plate, which was developed in 1 m LiCl, 0.5 mM formic acid. The amounts of [32P]orthophosphate released were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). The rates of ATP hydrolysis were determined in the linear range of reaction time and protein concentration dependence.

Helicase Substrates—The fork-like substrate, a 3′-P-5′ end-labeled 47-nt oligonucleotide 5′-TGGTTTTCCAGTCAGACATG-3′, was annealed to M13mp19 ssDNA, yielding a partial duplex with 17 bp, and 5′ and 3′ tails of 15 nt each. The substrate used to determine helicase polarity, a 33-nt oligonucleotide 5′-CAGACTGCTGATCCCGGGGATC-3′, was end-labeled at either the 5′ or 3′ end, annealed to M13mp19 ssDNA, and digested with SmaI as described (21). The long substrate, M13mp19 ssDNA primed with the 33-nt oligonucleotide above, was elongated using Klenow DNA polymerase as described (22).

DNA Helicase Assay—Unless stated otherwise, the reaction mixture (10 µl) consisted of 20 mM Tris-HCl (pH 7.5), 8 mM DTT, 1 mM MgCl2, 1 mM ATP, 20 mM KCl, 4% (w/v) sucrose, 80 µg/ml BSA, 1 ng of 32P-labeled helicase substrate (2000 cpm), and different amounts of helicase. The reaction mixture was incubated at 37 °C for 30 min and stopped by addition of 0.3% SDS, 10 mM EDTA, 5% glycerol, and 0.03% bromphenol blue (final concentration). The samples were analyzed by electrophoresis on 12% native polyacrylamide gels in 89 mM Tris borate, 2 mM EDTA. The gel was dried and autoradiographed. One unit of helicase activity was defined as the amount of enzyme that unwinds 1% of the substrate in 1 min at 37 °C in the linear range of enzyme concentration dependence.

Purification of Other Proteins—Recombinant human pol-prim was purified from insect cell extracts by immunoaffinity chromatography as described (16). Recombinant SV40 T antigen was expressed in insect cells infected with recombinant virus and purified by immunoaffinity chromatography as described (17). Recombinant human RPA was expressed in bacteria and purified as described (18). E. coli single-stranded DNA-binding protein was purified as described (19).

Expression of Recombinant FEN-1—Human FEN-1 cDNA in pET3d was kindly provided by A. Dutta (20). A recombinant baculovirus encoding human FEN-1 was constructed by inserting an NcoISpal fragment containing the FEN-1 cDNA into pVL1393 cut with NcoI and StuI, followed by co-transfection of the resulting pVL1393-FEN-1 construct with Baculogold DNA into Sf9 cells, as described above for the HDHB baculovirus cDNA-transcribed.

Immunoprecipitation—Extracts from insect cells infected with baculoviruses expressing tagged HDHB, pol-prim, or FEN-1 were incubated for 1 h at 4 °C on a rotating wheel with agarose beads coupled to T7 tag antibody (Novagen, Madison, WI), collected by centrifugation, and washed 5 times with lysis buffer. The eluate was diluted to 4-fold with Buffer Q (20 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM DTT, 0.5 mM EDTA, 0.01% Nonidet P-40) and loaded onto a 1 ml Mono Q column (Amersham Biosciences). Proteins were eluted with a 20-ml gradient of NaCl from 100 to 500 mM in Buffer Q and collected in 0.4-ml fractions. Protein concentration was determined by densitometric scanning of stained SDS-polyacrylamide gels (15). As protein standards, known amounts of bovine serum albumin (BSA) were loaded onto the same gel. The yield was generally about 0.5 mg of purified protein.

Microinjection of Synchronized Cells—HeLaS3 cells were arrested in G2/M with 50 µg/ml nocodazole (Sigma) for 16 h, as verified by flow
motifs I-VI in HDHB, E. coli any amino acid. Consensus residues of superfamily 1: sequence identity (*) between the helicase motifs of HDHB and recD. H9262 cyanate-conjugated goat anti-rabbit antibody (200 g/ml) (Jackson ImmunoResearch) was co-injected to identify the injected cells. Immediately after microinjection, bromodeoxyuridine (BrdUrd) was added to the medium at a final concentration of 3 µg/ml after microinjection, bromodeoxyuridine (BrdUrd) was added to the medium at a final concentration of 3 µg/ml. After 16 h at 37 °C, cells were fixed with 3% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 10% fetal calf serum. The cells were then immunostained with monoclonal mouse anti-BrdUrd primary antibody (1:10 dilution) (Amersham Biosciences) in the presence of benzonase (1:10 dilution) (Amersham Biosciences) in the presence of benzonase and stained with ethidium bromide.

RESULTS
cDNA Cloning and Expression of HDHB—A human cDNA with strong homology (65% identity and 75% similarity) to the cDNA sequence of mouse DNA helicase B, kindly made available by S. Tada prior to publication (9), was generated by using RT-PCR amplification and characterized (GenBank™ accession number AF319995). The cDNA sequence, HDHB, resides on human chromosome 12q13, and the open reading frame is distributed over 13 exons (25). The deduced amino acid sequence of HDHB contains 1087 residues with seven helicase consensus motifs (marked with boxes in Fig. 1A) that are characteristic of superfamily 1 helicases (10, 26). As in other superfamily 1 proteins and in dda helicase of bacteriophage T4 (Fig. 1B) (28). Overall, the predicted HDHB amino acid sequence is 29% identical and 45% similar to E. coli recD and 26% identical and 47% similar to dda. The deduced amino acid sequence of HDHB also harbors nine potential sites of phosphorylation by cyclin-dependent kinases. Seven of these sites are clustered at the C terminus (amino acid residues 967–1059), and the other two are in the N terminus (residues 5 and 119). Obvious orthologs of HDHB in the S. cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster, and Caenorhabditis elegans genomic sequence databases could not be identified.

Expression of HDHB mRNA was detected in a variety of human cell lines and tissues by RT-PCR and dot blot hybridization (Fig. 2). Relative expression levels of HDHB mRNA in different samples were quantitated by comparison of the HDHB hybridization signal with the amount of ribosomal RNA in the same sample of total RNA, whereby the value for testis RNA was set to 1. Bars indicate standard deviation in four independent experiments.

Enzymatic Activities of Recombinant HDHB—The homology between HDHB and mouse DNA helicase B (9) suggested that the enzymatic properties of HDHB might resemble those de-
scribed previously for DNA helicase B purified from murine cells (8, 13, 21, 29, 30). To test this prediction, recombinant HDHB was purified. For ease of purification and detection, a recombinant baculovirus was used to express wild type (wt) HDHB as a fusion protein with hexahistidine and T7 tags at the N terminus. Mutant forms of HDHB with an alanine in place of the conserved lysine in the Walker A motif (K481A, called mutant A) or glutamine in place of the conserved glutamate in the Walker B motif (E591Q, called mutant B) were also expressed. The recombinant proteins were purified by a two-step chromatography procedure and analyzed by denaturing PAGE and Coomassie staining (Fig. 3A) and by Western blotting with antibody against the T7 tag (not shown). The apparent molecular mass of HDHB of ~180 kDa was somewhat greater than expected from the deduced amino acid sequence and the extra residues comprising the N-terminal tags. The slow electrophoretic migration of recombinant HDHB may be caused by post-translational modification or by unusual amino acid sequences in the protein. Zone velocity centrifugation experiments with purified recombinant HDHB indicated a sedimentation coefficient of 6 S, suggesting a monomeric protein. 2

The enzymatic activity of recombinant wt HDHB was initially examined in ATPase assays in the presence of increasing amounts of ssDNA, double-stranded DNA, or polyuridylic acid (Fig. 3B). ATPase activity was very low in the absence of nucleic acid. However, even small amounts of ssDNA dramatically stimulated ATP hydrolysis by HDHB. Duplex DNA stimulated ATPase activity slightly, whereas the RNA polymer had no effect. ATP hydrolysis was dependent on the concentration of wt HDHB, but neither mutant form of HDHB had measurable ATPase activity (Fig. 3C). The ATPase turnover rate of wt HDHB in the presence of 100 ng of ssDNA was estimated to be about 280 mol of ATP per mol of HDHB per min. Helicase activity was tested using a fork-like DNA substrate with single-stranded 5’ and 3’ tails of 15 nucleotides each and a central 17-bp duplex region. Helicase activity was observed with wt HDHB but not with mutant A or B (Fig. 3D). These results indicate that recombinant HDHB has intrinsic single-stranded DNA-dependent ATPase and DNA helicase activities.

To further characterize the enzymatic activity of HDHB, the dependence of its unwinding activity on nucleotide concentration, divalent cations, and ionic strength was determined using a fork-like substrate. In the absence of ATP, unwinding activity was not detected, indicating that ATP was required. Helicase activity rose as the concentration of ATP was increased (Fig. 3A), but ATP concentrations higher than 10 mM inhibited the helicase activity (data not shown). Both ATP and dATP supported HDHB unwinding activity, but other ribo- and deoxyribonucleoside triphosphates were not utilized efficiently (Fig. 3B). Consistent with the notion that ATP hydrolysis is required for DNA unwinding by HDHB, the ATP analog ATPγS did not support unwinding activity (Fig. 4B). Helicase activity was detected with either Mg2+ or Mn2+, but not with other divalent cations, and was inhibited by KCl concentrations greater than 100 mM (data not shown).

The polarity of HDHB helicase activity was determined by using the DNA substrates diagrammed in Fig. 5A (21). Recombinant HDHB was found to efficiently displace the 3’-labeled 22-mer (Fig. 5A, lane 3). Conversely, unwinding and displacement of the 5’-labeled 14-mer was not observed (Fig. 5A, lane 7). As a control, SV40 T antigen, which has a 3’ to 5’ directionality (31–33), was assayed with the same substrates (Fig. 5A, lanes 2 and 6). Because the directionality of unwinding is defined by convention according to the DNA strand on which the enzyme translocates (12), the data demonstrate that HDHB has a 5’ to 3’ polarity of unwinding.

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2 P. Taneja and E. Fanning, unpublished data.
The helicase substrates used in Fig. 3, Fig. 4, and Fig. 5A contained a duplex region of only 14 to 22 bp, raising the question of whether HDHB was capable of unwinding substantially longer regions of duplex DNA. To address this question, a radiolabeled 33-mer oligonucleotide annealed to M13 ssDNA was elongated with DNA polymerase I Klenow fragment to yield a population of helicase substrate molecules with duplex regions of up to about 400 nucleotides in length (Fig. 5B, compare lane 1 with lane 5). In the presence of increasing amounts of HDHB, increasing amounts of radiolabeled products were generated (lanes 2–4). Interestingly, the relative amounts of radiolabeled products of different lengths corresponded closely to their relative amounts in the heat-denatured substrate (compare lanes 2–4 with lane 5) with no preference for shorter substrates. In addition, the data in Fig. 5, A and B indicate that HDHB unwinding activity does not require a fork-like DNA substrate.

**Functional Interactions of Recombinant HDHB with Human pol-prim and RPA**—Because DNA helicase B purified from mouse cells was shown to stimulate the activity of murine pol-prim (6, 7), recombinant HDHB was also expected to interact functionally with human pol-prim. Indeed, on an M13 ssDNA template, HDHB stimulated the synthesis of RNA primers of 8–10 nt by human pol-prim. Because murine DNA helicase B was reported to co-purify with pol-prim (34), it was important to confirm that no primase activity was detected with HDHB alone (lane 9). Heat treatment of HDHB abolished the stimulation of primase activity (compare lanes 3 and 10), indicating that native HDHB was required for stimulation of primase. In the presence of RPA, pol-prim synthesized few primers on M13 ssDNA template (Fig. 6B, compare lanes 3 and 4). However, small amounts of HDHB reversed this inhibition in a concentration-dependent manner (lanes 5–7). Heat-denatured HDHB was unable to stimulate primer synthesis in the presence of RPA (lane 8). HDHB did not detectably stimulate primer synthesis in the presence of E. coli single-strand DNA-binding protein (data not shown). The HDHB-mediated stimulation of primer synthesis in the presence of RPA was similar to that mediated by SV40 T antigen used as a positive control, except that larger amounts of T antigen were required to observe the stimulation (16) (data not shown).

The functional interactions of SV40 T antigen with pol-prim and RPA appear to depend on direct, specific physical associations among these proteins (35–42), raising the question of whether recombinant HDHB may also form complexes with pol-prim or RPA. To test this possibility, agarose beads coupled to T7 tag antibody were used to immunoprecipitate T7-tagged HDHB from extracts of insect cells co-expressing tagged HDHB and recombinant human pol-prim. Eluates from the beads were analyzed by Western blotting with antibodies against the two largest subunits of pol-prim (Fig. 7, A–C). When all four subunits of human pol-prim were co-expressed with wt HDHB (Fig. 7A, lane 2), mutant A (lane 3), or mutant B (lane 4), the p180 and p68 subunits co-precipitated with all three forms of HDHB. Interestingly, HDHB co-precipitated the full-length p180 but not the p180 degradation products observed in Western blots of the input cell extracts. No pol-prim subunits were detected in immunoprecipitates from extracts expressing pol-prim without the tagged HDHB (Fig. 7A, lanes 1, compare IP and input cell extracts).

SV40 T antigen appears to bind independently to all four

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**Fig. 4.** Nucleotide dependence of DNA unwinding by HDHB. A, helicase assays containing 30 fmol of purified wt HDHB and 6.25 fmol of fork-like substrate were carried out with the indicated concentrations of ATP. Unwound DNA was detected by native gel electrophoresis and phosphorimaging. B, standard helicase assays containing 30 fmol of purified HDHB were performed, except that 1 mM ATP was substituted by 100 μM of the indicated nucleotide. After 30 min at 37 °C, reaction products were analyzed as in A. Amounts of displaced oligonucleotide were expressed as a percentage of the starting substrate.

**Fig. 5.** Direction and efficiency of DNA unwinding by HDHB. A, the polarity of unwinding was tested in standard helicase assays, except that the fork-like substrate was substituted by either of those diagrammed here. An asterisk indicates the site of radiolabeling. Products were visualized using phosphorimaging. Lanes 1 and 5, no helicase; lanes 2 and 6, SV40 T antigen (600 ng); lanes 3 and 7, HDHB (10 ng); lanes 4 and 8, heat-denatured substrates. B, the ability of HDHB to unwind long duplex substrates was tested in standard helicase assays, except that 1 ng of the fork-like substrate was substituted by 8 ng of an M13-based substrate with duplex regions of various lengths. Products were separated by native gel electrophoresis, electrophoresed, and visualized using phosphorimaging. Lane 1, substrate (S); lanes 2–4, 10, 20, and 40 ng of HDHB; lane 5, heat-denatured substrate (HS).
Helicase-deficient HDHB Mutants Inhibit DNA Synthesis in Human Cells—The results in Figs. 3 and 7 indicate that HDHB mutants A and B lack detectable helicase activity but retain the ability to interact physically with pol-prim. Thus if these activities of HDHB are both required for DNA replication in human cells, the mutant proteins might interfere with the activity of endogenous wild type HDHB, causing a block or reduction in DNA synthesis. To assess this possibility, purified wild type or mutant forms of recombinant HDHB, mixed with rabbit immunoglobulin as a marker, were microinjected into the nuclei of human HeLa cells synchronized in G1 phase of the cell cycle. BrdUrd was added to the medium to monitor DNA synthesis. After 16 h of incubation, injected cultures were stained by indirect immunofluorescence to visualize BrdUrd synthesis (Fig. 9). HDHB mutant A protein injected in early G1 phase caused cells to arrest in late G1 or retard their entry into S phase. In addition, the data show that mutants A and B differ in their ability to inhibit S phase entry.

Regardless of when in G1 or S phase the cells were microinjected with wild type HDHB protein, no inhibition of DNA replication was observed (Fig. 9, compare HDHB wt and mock injected cells). When cells were injected with mutant B protein at 5 h after release from the nocodazole block, DNA synthesis was prevented in most of the injected cells (Fig. 9, filled circles). However, as G1 phase progressed, the cells gradually lost their sensitivity to inhibition by mutant B protein. Cells injected with mutant B protein at 9 to 10 h after release from G2/M or in S phase showed strong BrdUrd staining in most cells. Comparison of DNA synthesis in pulse-labeled uninjected cells and mutant B-injected cells as a function of time after release from the G2/M block suggests that loss of sensitivity to mutant B protein occurred about 4 to 5 h prior to detectable DNA synthesis (Fig. 9). HDHB mutant A protein injected in early G1 was less effective than mutant B in blocking DNA synthesis, and cells had lost their sensitivity to mutant A by 8–9 h after release from the G2/M block (Fig. 9, open diamonds). These results demonstrate that cells were susceptible to the effects of HDHB mutant B early in G1, but not late G1 or S phase, causing them to arrest in late G1 or retarding their entry into S phase. In addition, the data show that mutants A and B differ in their ability to inhibit S phase entry.
and precipitates (treated from cell extracts using T7 tag antibody agarose). The immunoblots (SDS-PAGE and Western blot using antibodies against p180, HDHB, together with baculoviruses encoding (tsFT848 cells expressing mouse DNA helicase B with thermosensitive phenotype (see Figs. 8 and 9). Similarly, tsFT848 cells expressing mouse DNA helicase B with thermosensitivity (29) would be consistent with the speculation that wild type HDHB in mediating priming by pol-prim on RPA-coated template strongly implicates HDHB as a mediator protein in one or more aspects of DNA metabolism that depend upon priming by pol-prim. Genetic studies have revealed essential functions of pol-prim in initiation of DNA replication, lagging strand DNA synthesis, telomere synthesis, and double-strand DNA break repair (4, 49–52). However, additional clues will be needed to distinguish the role of HDHB in these possible pathways.

Another clue to the physiological functions of HDHB is suggested by the cell cycle-dependent sensitivity to inhibition by mutant HDHB. The ability of HDHB mutant B to inhibit the G1/S transition when present during early G1 (Fig. 9) coincides with the timing of pre-replication complex assembly, suggesting that HDHB may associate with them (53). The observation that cells lose susceptibility to HDHB mutant B before late G1 (Fig. 9) would be consistent with the speculation that wild type HDHB assembles with pre-replication complexes in early G1, precluding the association of mutant B with the complex. If HDHB activity were required at replication forks during S phase, one would expect the onset of S phase to be retarded or prevented by helicase-deficient HDHB, as observed (see Figs. 8 and 9). However, the rates of fork elongation appeared to be similar in the tsFT848 mutant and parental cells at the restrictive temperature, arguing against an essential role of DNA helicase B in unwinding at the fork (8). Moreover, growing evidence that MCM2–7 serves as the helicase in initiation and at replication forks (53) and the observation that non-mammalian eukaryotic genomes lack an obvious HDHB ortholog suggest that HDHB may fulfill some other function. Nevertheless, it should be noted that some replication systems utilize two or more DNA helicases, e.g. gp41 and dda in bacteriophage T4 and UL9 and UL5 in Herpes simplex virus (54, 55).

The sequence homology of DNA helicase B with prokaryotic

\[ \text{DISCUSSION} \]

**HDHB is the Human Ortholog of Mouse DNA Helicase B**—Comparison of the deduced amino acid sequence of HDHB cDNA with that of mouse DNA helicase B cDNA (9) suggests that HDHB is closely related to DNA helicase B. Consistent with the sequence homology, preliminary characterization of the enzymatic properties of recombinant HDHB revealed similarities with DNA helicase B purified from mouse cells. Both proteins displayed robust ATPase activity dependent on single-stranded DNA, preferred ATP and dATP as substrates, and unwound several hundred base pairs of duplex DNA with 5’-3’ polarity (see Figs. 2–5 and Refs. 6, 8, 21, 29, and 30). No preference for a fork-like DNA substrate was noted for either helicase activity (see Fig. 5 and Refs. 6 and 21). In addition, both helicase activities were inhibited by salt concentrations greater than 100 mM, used either Mg²⁺ or Mn²⁺ to support helicase activity, and sedimented as monomers in zone velocity centrifugation (29, 44).³ Significantly, both helicases mediated the synthesis of RNA primers by pol-prim on single-stranded DNA template in the presence of mammalian RPA (see Fig. 6 and Refs. 6 and 7).

In addition to these similarities, analysis of helicase-deficient mutants of HDHB and mouse DNA helicase B suggests that both proteins function in a process needed for the onset of S phase. Introduction of a helicase-deficient HDHB protein into human cells in G1 inhibited the G1/S transition, suggesting a dominant-negative phenotype (see Figs. 8 and 9). Similarly, tsFT848 cells expressing mouse DNA helicase B with thermostable ATPase activity appeared to arrest at the G1/S transition at the restrictive temperature (8). The FM3A mouse mammary carcinoma cell line, from which the tsFT848 mutant was derived, is approximately tetraploid (45), suggesting that the mutation may have been dominant-negative at the restrictive temperature. Although mutant proteins that have a dominant-negative phenotype are commonly oligomeric, dominant-negative alleles of DNA helicase II of *E. coli*, a superfamily 1 member that is active as a monomer, have been described (46–48). The ability of HDHB mutant B to inhibit S phase onset more dramatically than mutant A (see Figs. 8 and 9) suggests that the two mutants differ in some property needed for dominance. HDHB wt and mouse DNA helicase B appear to bind strongly to single-stranded DNA in the absence of ATP but dissociate readily in the presence of 1 mM ATP (29).⁴ Interestingly, HDHB mutant A bound only weakly to ssDNA both with and without nucleotide, whereas mutant B bound better to ssDNA than the wild type and released it only partially when ATP was added.⁵ These observations lead us to speculate that HDHB mutant B, having bound to ssDNA, may bind ATP but not hydrolyze it, thereby blocking the catalytic cycle and subsequent DNA processing. Perhaps mutant A binds to DNA too weakly to block DNA processing efficiently. Alternatively, mutants A and B may differ in their ability to interact with an unknown protein partner. In summary, the similarities between the protein sequences, enzymatic properties, and mutant phenotypes of HDHB and mouse DNA helicase B support the conclusion that they are orthologous proteins.

What Are the Physiological Functions of HDHB and Mouse DNA Helicase B in DNA Metabolism?—Like SV40 T antigen, DNA helicase B interacts functionally with pol-prim and RPA (see Figs. 6 and Refs. 6 and 7), as well as physically (see Fig. 7 and Ref. 34). This striking similarity with T antigen provides one clue to possible functions of the cellular helicase. The potent activity of HDHB in mediating priming by pol-prim on RPA-coated template strongly implicates HDHB as a mediator protein in one or more aspects of DNA metabolism that depend upon priming by pol-prim. Genetic studies have revealed essential functions of pol-prim in initiation of DNA replication, lagging strand DNA synthesis, telomere synthesis, and double-strand DNA break repair (4, 49–52). However, additional clues will be needed to distinguish the role of HDHB in these possible pathways.

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⁴ Y. Wang, J. Gu, and E. Fanning, unpublished data.

⁵ Y. Wang, J. Gu, and E. Fanning, unpublished data.
recombination proteins recD and T4 dda provides a third possible clue to its functional role (see Fig. 1 and Ref. 9). If, like recD, DNA helicase B participates in initiating homologous DNA recombination during S phase, it might interact with pol-prim and RPA during reactivation of stalled or broken replication forks (56–59). However, if HDHB performs a recD-like function, one might expect it to associate with recBC-like proteins or with other nucleases (57). recBC orthologs have not been identified in the human genome (25), but recD homologs have been found in several other organisms that lack recBC homologs (57). By analogy with T4 dda, which can promote replication fork movement through a tightly bound protein molecule, DNA helicase B might rescue stalled forks by displacing the obstacle at the fork and then mediating new primer synthesis and reassembly of a replication complex (54, 60, 61). During G1 phase of the cell cycle, double-strand break repair is also thought to proceed by pathways that require participation of pol-prim (52). If HDHB functions through these pathways, defects in HDHB would be expected to delay or prevent double-strand break repair in G1, resulting in checkpoint-dependent arrest at G1/S. In summary, although the specific roles of HDHB in DNA metabolism remain to be elucidated, the work presented here and elsewhere (9) offers some useful clues and a foundation for future studies toward this goal.

Acknowledgments—We thank Shusuke Tada for communicating the cDNA sequence of mouse DNA helicase B prior to its publication, Anindya Dutta for FEN-1 antibody and cDNA, Shusuke Tada, FIG. 8. Microinjection of HDHB mutant proteins A and B into nuclei of G1 phase cells inhibits DNA synthesis. A, purified HDHB wt, mutant A, or mutant B protein as indicated was injected into the nuclei of HeLa-S3 cells in G1. Rabbit IgG was co-injected with HDHB samples to identify injected cells. After microinjection, BrdUrd was added to the medium for 16–18 h. Cells were then fixed, permeabilized, and stained by immunofluorescence to detect BrdUrd incorporation and the co-injected rabbit IgG. Nuclear DNA was visualized by Hoechst dye. B, BrdUrd staining was qualitatively evaluated as none, partial, or strong in 120 uninjected cells, in 150 cells injected with buffer (Mock), HDHB wt (150 cells), HDHB mutant A (HDHB MutA; 100 cells) and HDHB mutant B (HDHB MutB; 150 cells). The average percentage of cells in each category was determined in three independent microinjection experiments (bars at the top of each column indicate standard deviation).

FIG. 9. Cells in early G1, but not late G1 or S phase, are sensitive to HDHB mutants deficient in DNA helicase activity. HDHB wt (filled squares), mutant A (open diamond), mutant B (filled circles) protein, or IgG-containing injection buffer (open squares) was microinjected into HeLa cell nuclei at the indicated times after release from a nocodazole block. For the S phase time point (S), cells were injected 1 h after release from thymidine block. BrdUrd was added to the medium after injection, and incorporation was detected by immunostaining. The percentage of uninjected cells in S phase at the indicated times after release from the block was determined by BrdUrd pulse labeling (open circles). Between 80 and 100 cells were evaluated at each time point, and the standard deviation is indicated by the bars on each point.
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