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Contribution of DEAH-box protein DHX16 in human pre-mRNA splicing

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Abstract

Studies of mammalian splicing factors are often focused on small nuclear ribonucleoproteins or regulatory RNA-binding proteins, such as hnRNP (heterogeneous nuclear ribonucleoprotein) and SR proteins (serine/arginine-rich proteins); however, much less is known about the contribution of DExD/H-box proteins or RNA helicases in mammalian pre-mRNA splicing. The human DEAH-box protein DHX16 [also known as DBP2 (DEAD-box protein 2)], is homologous with *Caenorhabditis elegans* Mog-4, *Schizosaccharomyces pombe* Prp8 and *Saccharomyces cerevisiae* Prp2. In the present study, we show that DHX16 is required for pre-mRNA splicing after the formation of a pre-catalytic spliceosome. We found that anti-DHX16 antiserum inhibited the splicing reaction *in vitro* and the antibody immunoprecipitated pre-mRNA, splicing intermediates and spliceosomal small nuclear RNAs. Cells that expressed DHX16 that had a mutation in the helicase domain accumulated unspliced intron-containing minigene transcripts. Nuclear extracts isolated from the dominant-negative DHX16-G724N-expressing cells formed splicing complex B, but were impaired in splicing. Adding extracts containing DHX16-G724N or DHX16-S552L mutant proteins to HeLa cell nuclear extracts resulted in reduced splicing, indicating that the mutant protein directly inhibited splicing *in vitro*. Therefore our results show that DHX16 is needed for human pre-mRNA splicing at a step analogous to that mediated by the *S. cerevisiae* spliceosomal ATPase Prp2.

Keywords

DExD/H-box protein 16 (DHX16); pre-mRNA splicing; *Saccharomyces cerevisiae* Prp2; spliceosome

INTRODUCTION

Nuclear pre-mRNA splicing consists of two simple transesterification reactions, but requires one of the most complex macromolecular complexes, the spliceosome, to complete them [1]. The spliceosome is composed of five snRNAs (small nuclear RNAs) and nearly 100

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AUTHOR CONTRIBUTION

Marieta Gencheva, Mitsuo Kato and Alain Newo performed experiments and all authors contributed to experimental design, ideas and manuscript writing. Ren-Jang Lin put together the final version of the manuscript for submission.

proteins, many of which are either associated with the snRNAs or bound to the intron or exon sequences [2]. The dynamic interactions among the snRNAs, proteins and pre-mRNA substrate are mediated at least in part by DExD/H-box proteins, which are RNA helicases or RNA-dependent ATPases [3]. Although the role of DExD/H-box proteins has been characterized in yeast [4], much less is known about their contribution to human pre-mRNA splicing.

Eight DExD/H-box proteins are required for pre-mRNA splicing in *Saccharomyces cerevisiae*: Prp2, Prp5, Prp16, Prp22, Prp28, Prp43, Sub2 and Brr2 [5]. Their mammalian counterparts are called DDXs for DExD-box or DHXs for DExH-box proteins [6]. Proteins in this family have a well-conserved helicase domain, a conserved C-terminal domain and a divergent N-terminal domain [7]. Owing to the high conservation, assigning functional orthologues among different species based solely on sequence alignments can be difficult.

Human DHX16 was initially identified as DBP2 (DEAD-box protein 2) in a search for human DEAH-box RNA helicases [8]. The gene resides on chromosome 6p21.3 in the MHC region, which is linked to several malignant and autoimmune diseases. The helicase domain of DHX16 has >72% identity to *Schizosaccharomyces pombe* Cdc28/Prp8, a protein involved in cell-cycle progression and pre-mRNA splicing [9], and human DHX16 can rescue a temperature-sensitive *S. pombe prp8-1* mutant, albeit inefficiently [8], supporting the idea that DHX16 is a functional homologue of Cdc28/Prp8. Another orthologue, *Caenorhabditis elegans* Mog-4, exhibits 53.9% identity with DHX16. Mog-4 plays a role in the hermaphrodite sperm/oocyte switch, and knockdown of Mog-4 is embryonic lethal [10]. A function in splicing has not been proposed for Mog-4, but Mog-4 is needed for a posttranscriptional repression of the *fem-3* sex-determining gene through an interaction with the 3'-UTR (untranslated region) of *fem-3* [11]. The *S. cerevisiae* orthologue for DHX16 is proposed to be Prp2 [10], as DHX16 is found in spliceosome preparations from HeLa cells [12–15]; however, a BLAST search on the *S. cerevisiae* database (<http://www.yeastgenome.org>) identified Prp22 as the likely orthologue, whereas the UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene>) identified Prp43. Therefore the role of DHX16 in human pre-mRNA splicing still needs to be defined.

In the present study, we investigated the contribution of DHX16 in human pre-mRNA splicing. We used an anti-DHX16 antibody to show that DHX16 associates with the spliceosome, is needed for the first transesterification and did not completely dissociate from the spliceosome, even after the second transesterification. We generated several DHX16 helicase-domain-mutants and show that they inhibit splicing of minigene transcripts *in vivo* and *in vitro*. Our results indicate that DHX16 contributes to human pre-mRNA splicing before the first transesterification and shares characteristics with *S. cerevisiae* Prp2.

EXPERIMENTAL

Plasmids

The open reading frame of DHX16 was amplified from pBluescript II SK+ KIAA0577 [16] by PCR using high-fidelity Pfu-Turbo polymerase (Stratagene) with primers containing an EcoRI and an XhoI site (Supplementary Table S1 at <http://www.BiochemJ.org/bj/429/bj4290025add.htm>) and inserted into pCR (Invitrogen). The K428A, D520A, H523D, S552L and G724N mutations were introduced into DHX16 either by using a site-directed mutagenesis kit (Stratagene) or by following the protocol described by Kunkel et al. [17]. FLAG–DHX16 fusion proteins were constructed by inserting a FLAG tag (MDYKDDDDK) before the second codon of DHX16 via a PCR strategy. The FLAG–DHX16 fragments were cloned between the EcoRI and XhoI sites of pCDNA3.1 (Invitrogen). EGFP (enhanced green fluorescent protein)–DHX16 was made by

inserting the EcoRI/XhoI fragment of DHX16 between the EcoRI and SalI sites of pEGFP-C2 (Clontech) such that EGFP was placed at the N-terminus of DHX16. All constructs were sequenced for verification. FLAG–DHX16-G724N (used in Figure 6) contained an additional E484A mutation outside the conserved helicase motif; however, the E484A mutation did not have an observable effect in the *in vitro* or *in vivo* splicing assays. pL53In contains a minigene with an intron (MoBiTec).

Cell lines and transfection

HeLa, HEK (human embryonic kidney)-293 and HEK-293T cells [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)] were cultured in DMEM (Dulbecco's modified Eagle's medium; Irvine Scientific) supplemented with 10% (v/v) FBS (fetal bovine serum, HyClone) under a 5% CO₂ atmosphere at 37°C. Cells (8×10^5) were transfected with 2–3 µg of plasmid DNA by Lipofectamine™ 2000-mediated transfection (Invitrogen) on a dish, or in suspension and then plated. Calcium phosphate-mediated transfection of HEK-293T cells (60–70% confluent) was performed on plates. Briefly, a 500 µl solution containing 250 mM CaCl₂ and 20 µg of plasmid DNA was added dropwise with constant mixing to an equal volume of buffer (50 mM HEPES, pH 7.1, containing 280 mM NaCl and 1.5 mM Na₂HPO₄). After a 30 min incubation at room temperature (22°C), the mixture was added dropwise to the medium covering the cells and the medium was changed after 18 h.

Antibody production, Western blotting and immunostaining

A peptide (CPKKIGKTREELG) from the C-terminus of DHX16 was synthesized in the Peptide Synthesis Facility at City of Hope, conjugated to keyhole limpet haemocyanin (Pierce), and injected into two rabbits. The animal experiments were performed by certified personnel at the City of Hope Animal Resources Center following the guidelines approved by the Institutional Animal Care and Use Committee (IUCUC no. 93023). The antibody was precipitated from the rabbit serum in saturated ammonium sulfate and resuspended in PBS [18]. To purify the peptide-specific anti-DHX16 antibody, the precipitated antibody was applied to a column of Affi-Gel 10 (Bio-Rad Laboratories) conjugated to the peptide used for immunization. The bound antibody was eluted with 100 mM glycine, pH 3, collected in 1 M Tris/HCl, pH 8, and dialysed against PBS.

For total protein extraction, cells were lysed in RIPA buffer [10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% (v/v) NP-40 (Nonidet P40), 1% (w/v) sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM DTT (dithiothreitol), 1 mM PMSF and 100 units/ml benzonase] or in TNE buffer [10 mM Tris/HCl, pH 8, 1% (v/v) NP-40, 150 mM NaCl and 1 mM EDTA] on ice for 20 min and cleared by centrifugation as described previously [19].

Protein lysates (10–20 µg) were resolved by SDS/PAGE (8% or 10% gels) and proteins were transferred on to PVDF membranes (Millipore). Antibodies used for Western blotting were: rabbit anti-DHX16, anti-TOPOII (topoisomerase type II)-β (Sigma–Aldrich), anti-tubulin (Sigma–Aldrich) and anti-FLAG–M2 (Sigma–Aldrich) antibody, and mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Ambion) antibody. SuperSignal West Pico chemiluminescent substrate (Pierce) was used for detection of HRP (horseradish peroxidase)-conjugated secondary antibodies. To re-probe, the antibody from the first probing was stripped from the membrane in 0.2 M glycine/HCl, pH 2.5 and 0.1% SDS.

For immunostaining, HeLa cells were grown on coverslips and fixed with 4% (w/v) paraformaldehyde for 20 min on ice. After two washes in PBS, cells were incubated with the primary antibody for 1 h on ice. The anti-SC35 antibody was a mouse monoclonal antibody and the anti-DHX16 antibody was a rabbit polyclonal antibody. The secondary antibody

used was anti-(mouse Ig)–Cy2 (carbocyanine) or anti-(rabbit-Ig)–Cy5 (indocarbocyanine). DAPI (4',6-diamidino-2-phenylindole; 1 µg/ml) was added to the final PBS wash (for a 5 min duration). Images were collected via fluorescence microscopy.

Preparation of nuclear and cytoplasmic fractions

HeLa cells (1.2×10^7 cells) were harvested, washed with PBS, and resuspended in one PCV (packed cell volume) of buffer A (10 mM Hepes/KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 1 mM DTT) on ice for 15 min. The swollen cells were passed through a 25-gauge needle five times and the cell lysate was centrifuged at 20000 g for 20 s; the supernatant was saved as the cytoplasmic fraction. The pellet was washed with 1 PCV of buffer A, resuspended in a 2/3 PCV of buffer C [20 mM Hepes/KOH, pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 420 mM NaCl, 0.5mM PMSF and 1 mM DTT] at 4°C for 30 min and centrifuged at 20000 g for 5 min; the supernatant was recovered as the nuclear fraction [20].

Whole-cell extract and nuclear extract for *in vitro* splicing assays

HEK-293T cells were transfected with plasmid DNA on 10-cm-diameter plates by calcium-phosphate-mediated transfection. At 36 h after transfection, cells were harvested (500 g for 1 min), washed in PBS and resuspended in 200 µl of ice-cold buffer E [20 mM Hepes/KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% (v/v) glycerol and 1 mM DTT]. Cells were lysed by sonication for 20 s (in 5 s pulses followed by 30 s rests) at 30% amplitude in a Fisher Scientific Model 500 Ultrasonic Dismembrator. The lysate was centrifuged (20000 g for 20 min at 4°C) and the supernatant was saved as whole-cell extract [21].

Standard HeLa nuclear extracts were prepared from 5×10^9 HeLaS3 cells (National Cell Culture Center) as described previously [22,23]. For small scale extractions, 5×10^7 cells were transfected with 80 µg of plasmid using Lipofectamine™ 2000, harvested 72 h after transfection and subjected to nuclear extract preparation [20].

In vitro splicing and spliceosomal complex assays

BamHI-digested pRG1 (1.5 µg), encoding a modified MINX (miniature wild-type) pre-mRNA as described previously [24], was transcribed in a 25 µl reaction containing: 2 mM m7G(5')ppp(5')G cap analogue, 125 µM ATP, 125 µM CTP, 125 µM GTP, 10 µM UTP, 10 mM DTT, 20 units of SP6 RNA polymerase, 30 µCi of [α -³²P]UTP (800 Ci/mmol) and buffer (New England Biolabs). BamHI-digested AdMLPar plasmid [25] was transcribed in: 2.0 mM m7G(5')ppp(5')G cap analogue, 0.5 mM ATP, 0.5 mM CTP, 0.1 mM GTP, 0.1 mM UTP, 10 mM DTT, 2 units/µl T7 polymerase, 0.4 units/µl RNasin, 1.2 µCi/µl [α -³²P]UTP and buffer (New England Biolabs). Transcripts were subjected to electrophoresis on a 6% polyacrylamide/urea gel, eluted from the gel slice in 0.5 M NH₄OAc, 0.1% SDS and 1 mM EDTA, and ethanol-precipitated with 10–20 µg of glycogen.

Labelled transcript (10000–20000 c.p.m.) was used in a 25-µl splicing reaction [26] containing: 40–50% extract, 0.5 mM ATP, 3.2 mM MgCl₂, 20 mM creatine phosphate, 20 mM Hepes/KOH, pH 7.6, 2.6% (v/v) polyvinyl alcohol and 0.4 units/µl RNasin at 30°C. The reaction was stopped by adding a solution of 0.3 M NaOAc, pH 5.2, containing 0.1% SDS. After phenol extraction, RNA was ethanol-precipitated with 10–20 µg of glycogen, resuspended and resolved on a 13% polyacrylamide/8 M urea gel (19:1). The gel was imaged by autoradiography or with a PhosphorImager (Typhoon 9000; GE Healthcare), and analysed with ImageQuant TL software (GE Healthcare).

For complex assays, splicing reactions were stopped by adding 0.1 volumes of 10× heparin loading dye [6.5 mg/ml heparin sulfate, 40% (v/v) glycerol, 0.5% Bromophenol Blue, 0.5%

Xylene Cyanol, 1 × TBE (45 mM Tris/borate and 1 mM EDTA)] and resolved on a 2% low-melting agarose gel containing 50 mM Tris and 50 mM glycine for approx. 6 h at 70 V [27]. Gels were fixed, dried and imaged by autoradiography or with a PhosphorImager.

Antibody inhibition and immunodepletion of DHX16 from nuclear extract

To test the effect of the anti-DHX16 antibody on splicing, antiserum or pre-immune serum (16 µg of protein equivalent) was added to the splicing mixture without pre-mRNA and incubated at 30°C for 20 min. ³²P-labelled pre-mRNA was then added to initiate the reaction. For the peptide-rescue experiment, 15 µg of antiserum or pre-immune serum was incubated at 30°C for 20 min with 600 ng of peptide before being added to the splicing mixture.

To deplete DHX16, 15 µl of anti-DHX16 antibody in TBS (Tris-buffered saline; 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl) plus 0.01% NP-40 and 0.5 mg/ml BSA was incubated for 2 h at 4°C with 25 µl of Protein A–Sepharose beads (Amersham Bioscience). The beads were then washed with TBS followed by buffer D [20 mM Hepes/KOH, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 1 mM DTT]. The antibody-bound beads were incubated for 2–3 h at 4°C with 100 µl of HeLa nuclear extract and then removed by centrifugation (1000 *g* for 1 min).

Immunoprecipitation and Northern blotting

Antibody or serum was incubated for 1 h at 4°C with Protein A–Sepharose (Sigma–Aldrich) and washed with immunoprecipitation buffer (20 mM Hepes, pH 7.9, 150 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT and 0.05% NP-40). An equal volume of heparin (1 mg/ml) was added to the splicing reaction mixtures (25 µl) and incubated for 5 min at 30°C. The mixture was incubated for a further 2 h at 4°C with 20 µl of Protein A–Sepharose beads coated with antibody, washed with immunoprecipitation buffer and eluted with peptide (50 µl of 0.6 mg/ml) at 4°C for 40 min. RNA was isolated from the eluate by phenol extraction and ethanol precipitation.

For Northern blot analysis, isolated RNA was separated on a 8% polyacrylamide/7.5 M urea gel and transferred on to membrane (NytranN; S&S) (1 mA/cm², 90 min, in 12 mM Tris/HCl, pH 6.8, containing 6 mM NaOAc and 0.3 mM EDTA) [28] using a Trans-Blot transfer cell (Bio-Rad Laboratories). RNA on the membrane was hybridized at 65°C overnight with ³²P-labelled antisense RNA of U1, U2, U4, U5 and U6 transcribed from plasmids pSPU1, pSPU2, pSPU4, pSPU5 and pSPU6 respectively by SP6 RNA polymerase. The membrane was washed with 0.1 × SSC (1 × SSC 0.15 M NaCl/0.015 M sodium citrate)/0.5% SDS at 65°C for 1 h and the signals were detected by autoradiography or with a PhosphorImager.

RNA isolation and RT (reverse transcription)–PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen). RNA was incubated for 15 min at 37°C with 5 units of DNase I (Roche) and then chloroform-extracted. cDNA was synthesized by RT of 5 µg of RNA using random hexamers (New England Biolabs) and Superscript II (Invitrogen). A 0.01 × volume of the RT reaction was used for PCR (95°C for 5 min, then 28–30 cycles of 20 s at 95°C, 20 s at 62°C and 30 s at 72°C, followed by a final extension at 72°C for 7 min) with the indicated primers (Supplementary Table S1).

RESULTS

DHX16 localizes in the nucleus

To determine the cellular localization of DHX16, we produced rabbit polyclonal antibodies against the DHX16 C-terminal peptide, PKKIGKTREELG. An approx. 120 kDa protein was detected by peptide-purified antibodies in Western blot analysis of whole cell lysates from HEK-293 cells (Figure 1A), consistent with the 119 kDa calculated molecular mass of DHX16. We then used the antibody to determine the cellular localization of DHX16 in cell fractions and fixed cells. We separated HeLa cell lysates into nuclear and cytoplasmic fractions and performed Western blot analysis. DHX16 was detected in the nuclear fraction, as was the nuclear protein DNA TOPOII- β , whereas tubulin, as expected, was found mostly in the cytoplasmic fraction (Figure 1B). We also stained fixed HeLa cells with antibodies against DHX16 and SR protein SC35, which is a splicing factor and widely used nuclear speckle marker; the anti-SC35 antibody revealed nuclear speckles, where many spliceosomal proteins localize, whereas anti-DHX16 staining was predominantly in the nuclei (Figure 1C). Further examination of the SC35 and DHX16 immunostaining revealed that the two signals overlapped, but the DHX16 staining was more broadly distributed in the nucleoplasm (Figure 1D). We also made an EGFP-DHX16 construct and found the fusion protein localized to the nucleus (results not shown), similar to the observations reported previously [8]. Therefore DHX16 appears to localize to the nucleoplasm and nuclear speckles, consistent with its proposed role in pre-mRNA splicing.

Anti-DHX16 antibody inhibits pre-mRNA splicing in HeLa nuclear extracts

To test whether DHX16 is required for splicing reactions *in vitro*, we added the anti-DHX16 antiserum to HeLa cell nuclear extracts. Western blot analysis showed that the antiserum reacted with the 120 kDa protein in the HeLa extract, whereas the pre-immune serum did not (Figure 2A). Reaction mixtures were assembled in the presence of anti- or pre-immune serum and ^{32}P -labelled MINX pre-mRNA was added to the reactions to allow splicing to take place for 0, 30, 60 or 90 min. RNA was then extracted and analysed by denaturing gel electrophoresis. The anti-DHX16 antiserum inhibited splicing as neither the spliced products nor intermediates were detected, whereas splicing occurred in the presence of pre-immune serum (Figure 2B). As yeast extracts lacking active Prp2 can assemble a pre-catalytic spliceosome [29], we also analysed the effect of the anti-DHX16 antiserum on spliceosome formation by native gel electrophoresis. The reaction mixtures containing HeLa nuclear extract and antiserum were incubated for 0, 5, 10, 20 or 40 min, and resolved on a native gel. The formation of splicing complexes A and B was not affected by the antiserum (Figure 2C), even though the splicing reaction was inhibited. To verify the specificity of splicing inhibition, we pre-incubated the antiserum with the peptide antigen prior to addition to the splicing reaction, which reversed the splicing inhibition (i.e. splicing occurred) (Figure 2D, α -DHX containing lanes).

To test further the requirement of DHX16 for splicing, we also analysed the splicing reaction in DHX16-depleted nuclear extract. HeLa nuclear extracts were incubated with or without the peptide-purified anti-DHX16 antibody conjugated to Protein A beads, and splicing was analysed on denaturing gels and formation of splicing complexes was analysed by non-denaturing gels. Western blotting showed that DHX16 was depleted from the extract (Figure 3A, Δ NE). An *in vitro* splicing reaction with AdML (adenovirus major late) pre-mRNA substrate and the immunodepleted extract exhibited no splicing activity (Figure 3B), but could still assemble spliceosomal complex B (Figure 3C). Taken together, these results indicate that DHX16 is involved in pre-mRNA splicing *in vitro* and that HeLa nuclear extracts assembled a catalytically inactive spliceosomal complex when DHX16 was inactivated or removed by an anti-DHX16 antibody.

DHX16 associates with pre-mRNA, splicing intermediates and snRNA during the splicing reaction

To investigate whether DHX16 is associated with the spliceosome, we used an anti-DHX16 antibody to precipitate the protein and its associated complexes during the splicing reaction. HeLa nuclear extracts were incubated with ³²P-labelled MINX pre-mRNA under splicing conditions for 0, 10, 20 or 60 min (Figure 4A, left-hand panel). At the indicated times the reaction mixtures were immunoprecipitated with the peptide-purified anti-DHX16 antibody and eluted with the peptide antigen. RNA was isolated from the eluate and analysed on denaturing gels (Figure 4A, middle panel). A set of immunoprecipitation reactions using the pre-immune serum was also carried out for comparison (Figure 4A, right-hand panel). Pre-mRNA was associated with DHX16 from 10 min to 60 min, whereas the excised exon 1 and intron–exon 2 lariat intermediates associated with DHX16 at the 20 and 60 min time points (Figure 4A, middle panel). Small amounts of intron and spliced exons were also present in the immunoprecipitated samples. These results indicate that DHX16 associates with the spliceosome before the first transesterification, remains associated with the spliceosome after the first step, and probably even after the second transesterification, suggesting that human DHX16 may have a function in the later stage of pre-mRNA splicing. Protein MS has shown that DHX16 is present in purified spliceosomal complex B, as well as in complex C (see the Introduction section). Thus this is an interesting difference between human DHX16 and yeast Prp2, because yeast Prp2 activates the spliceosome prior to the first step and is not involved in the later stage of the splicing reaction [30].

To verify that DHX16 was associated with the spliceosome during the splicing reaction, the immunoprecipitate was analysed by Northern blot for the presence of spliceosomal snRNAs (Figure 4B). By comparing the control immunoprecipitation with that using the pre-immune serum (Figure 4B), it was found that the U2, U4, U5 and U6 snRNAs were specifically associated with DHX16 after 10–60 min incubations (Figure 4B, left-hand panel). U1 snRNA was also found in the immunoprecipitates, but it was not clear whether the signal was specific. Nonetheless, the presence of four snRNAs in the DHX16 immunoprecipitates indicated that DHX16 associated with the pre-mRNA or splicing intermediates via the spliceosome.

Helicase domain mutants of DHX16 block splicing of minigene transcripts

To study further the role of DHX16 in splicing, we created DHX16 mutants by mutating various amino acids in the conserved helicase domain: K428A, D520A, H523D, S552L and G724N (Figure 5A). The latter three mutations are analogous to mutations in yeast Prp2 (H349D, S378L and G551N) that result in diminished ATPase activity and a dominant-negative phenotype [31,32]. We transfected HEK-293 cells with a mutant plasmid, a wild-type plasmid or the vector plasmid, along with an intron-containing minigene plasmid, pL53In. Western blot analysis of the transfected cell lysates, using the anti-DHX16 antibody, indicated expression of DHX16 from the transfected plasmid (Figure 5B). We then isolated total RNA and performed RT-PCR to detect unspliced and spliced minigene RNA. Unspliced pL53In transcript was detected in cells expressing DHX16-K428A, -D520A, -S552L and -G724N, a very small amount of unspliced RNA was detected in cells that expressed DHX16-H523D, and little or no unspliced transcript was detected in cells transfected with the wild-type or vector control (Figure 5B). The weak dominant-negative effect of the H523D mutation was somewhat unexpected, because an equivalent mutation in yeast Prp2 (H349D) is quite effective in inhibiting splicing [32]. Nevertheless, overproducing a DHX16 mutant protein with an altered conserved amino acid in motif I (K428A), II (D520A), III (S552L) or VI (G724N) (Figure 5A) resulted in accumulation of unspliced minigene transcript, suggesting that pre-mRNA splicing, as detected by a model intron-containing minigene, was impaired when DHX16 function was interrupted *in vivo*.

Nuclear extracts from mutant DHX16-G724N-expressing cells are impaired in splicing and accumulate spliceosome complex B

To analyse further the effect of the mutant protein on splicing, we prepared nuclear extracts from cells transfected with a mutant or wild-type DHX16 construct. We chose to study the DHX16-G724N mutant because of its robust inhibition of minigene transcript splicing (Figure 5C). HEK-293 cells were transfected with a plasmid carrying FLAG-tagged wild-type DHX16, FLAG-tagged DHX16-G724N or EGFP-tagged DHX16-G724N. Nuclear extracts were then prepared from transfected and untransfected cells. Western blot analysis showed that the transfected wild-type and mutant DHX16 proteins were expressed at a higher level than the native protein (Figure 6A). When assayed for *in vitro* splicing activity, nuclear extracts derived from the mutant DHX16-expressing cells had much less splicing activity than extracts from untransfected or wild-type DHX16-expressing cells (Figure 6B). The extracts containing DHX16-G724N failed to splice the MINX (Figure 6B, left-hand panel) or AdML (Figure 6B, right-hand panel) pre-mRNA substrates, indicating a general reduction in splicing activity in those extracts.

To analyse formation of the splicing complex, we resolved aliquots of the *in vitro* splicing reactions carried out in extracts from wild-type-DHX16-transfected cells and EGFP-DHX16-G724N-transfected cells on a native agarose gel (Figure 6C). Both wild-type and mutant extracts formed complexes A and B. The kinetics of complex A formation were similar between the wild-type and mutant extracts, but the mutant extract produced more complex B at later time points (Figure 6C). Therefore nuclear extracts isolated from DHX16-G724N-expressing cells are capable of assembling spliceosomal complex B, but are not effective in carrying out the transesterification reactions. This is consistent with DHX16 having a function after spliceosome formation and prior to the first catalytic step of splicing, similar to yeast Prp2 protein [30].

The weak activity in mutant extracts could be due to inhibition of the splicing reaction by the mutant protein *in vitro* or a lack of another late splicing factor in the extract (e.g. if there was less mRNA encoding a late splicing factor due to splicing inhibition by DHX16-G724N in the transfected cells). To test this, we isolated whole-cell extracts from cells that expressed mutant DHX16 and mixed them with the standard HeLa nuclear splicing extracts. If splicing activity was reduced beyond a simple dilution effect, we would conclude that the weak splicing activity shown in Figure 6 was caused by dominant inhibition by DHX16-G724N *in vitro*. The mixing experiment indicated that mutant DHX16 proteins, such as DHX16-G724N and DHX16-S552L, inhibited splicing *in trans* (see the Supplementary Results section and Supplementary Figure S1 at <http://www.BiochemJ.org/bj/429/bj4290025add.htm>). Thus the weak splicing activity *in vitro* (Figure 6) and *in vivo* (Figure 5) most probably resulted from direct participation of the mutant protein in splicing.

DISCUSSION

By using antibodies and helicase domain mutants, we have shown that DHX16 contributes to human pre-mRNA splicing prior to first transesterification reaction. A pre-catalytic spliceosome, splicing complex B, was stalled when DHX16 function was inhibited. Although human DHX16 has been suggested to be a *S. cerevisiae* Prp22 homologue [6], our results indicate that DHX16 is probably a functional orthologue of *S. cerevisiae* Prp2. As DHX16 complements *S. pombe cdc28/prp8* double-mutants that are defective in pre-mRNA splicing [8], those studies [6,8] further argued that *S. pombe* Cdc28/Prp8 is likely to be a splicing complex B helicase. Thus the requirement of a DEAH-box protein to activate a pre-catalytic spliceosome for the first transesterification reaction in pre-mRNA splicing [30] appears to be evolutionarily conserved.

Similarity between DHX16 and *S. cerevisiae* Prp2 resides in the helicase and C-terminal domains; there is essentially no similarity in their N-terminal regions. The N-terminal region of DHX16 is enriched in lysine, glutamate and arginine residues, and a similar KER domain enriched in these residues was initially found in CAF-I (chromatin assembly factor I) as a histone-binding domain [33]. Human DHX38 (a homologue of *S. cerevisiae* PRP16) has an N-terminal region enriched in arginine, glutamate, aspartate and serine residues, which is not found in *S. cerevisiae* Prp16 [34]. In addition, the N-terminal region of human DHX8 (a homologue of *S. cerevisiae* Prp22) is enriched in arginine and serine residues, which is not found in *S. cerevisiae* Prp22 [35]. These unique N-terminal regions may play an important role in human mRNA splicing and may contribute to a potential difference in splicing regulation between humans and *S. cerevisiae*. In fact, full-length DHX38 cannot rescue *S. cerevisiae* with a *prp16* deletion; only a chimaeric construct containing an *S. cerevisiae* N-terminus with human helicase and C-terminal domains can rescue the deletion [34]. We found that DHX16 did not complement *S. cerevisiae prp2* temperature-sensitive or deletion mutants (M. Kato and R. J. Lin, unpublished work), suggesting that the N-terminal domains of DHX16 and Prp2 are not compatible. Interesting future studies could elucidate the unique function of the KER domain in DHX16 function.

Anti-DHX16 antibody immunoprecipitated splicing intermediates, as well as some spliced products (Figure 4), indicating that DHX16 remained associated with the spliceosome after the first transesterification. However, *S. cerevisiae* Prp2 exits the spliceosome after the first transesterification [36,37]. It is possible that DHX16 remains on the human spliceosome because it has a more stable interaction with a late spliceosomal factor such as GPKOW (G-patch domain and KOW motifs), which is found in splicing complex C and could be a homologue of yeast Spp2 [15]. Yeast Prp2 interacts with Spp2 and this interaction is essential for splicing [37,38]. We are currently investigating the interaction between DHX16 and GPKOW and whether their interaction plays a role in keeping DHX16 on the spliceosome. Furthermore, it is of interest to determine whether DHX16 plays an additional role in splicing after the first transesterification.

Unspliced intron-containing minigene transcripts were detected when cells overexpressed a dominant-negative DHX16 mutant, either by transient transfection (Figure 5) or by inducible stable transfection (M. Kato and R. J. Lin, unpublished work). This is consistent with a requirement for DHX16 before the first transesterification. We have also identified, by genomic tiling microarray, several endogenous transcripts that were unspliced in mutant-DHX16-expressing cells (M. Gencheva and R. J. Lin, unpublished work). It appears that expressing mutant DHX16 in a human cell results in accumulating intron-containing pre-mRNAs. This is analogous to yeast *prp2* mutants accumulating spliceosomes that contained unspliced pre-mRNA *in vivo* [31,32,39]. Further characterization of DHX16 shall aid in the understanding of how a human spliceosome becomes active in splicing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used:

DHX	DExH-box
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

GPKOW	G-patch domain and KOW motifs
HEK	human embryonic kidney
HEK-293T cells	HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)
hnRNP	heterogeneous nuclear ribonucleoprotein
NP-40	Nonidet P40
PCV	packed cell volume
RT	reverse transcription
snRNA	small nuclear RNA
SR protein	serine-arginine rich protein
TBS	Tris-buffered saline
TOPOII	Type II topoisomerase.

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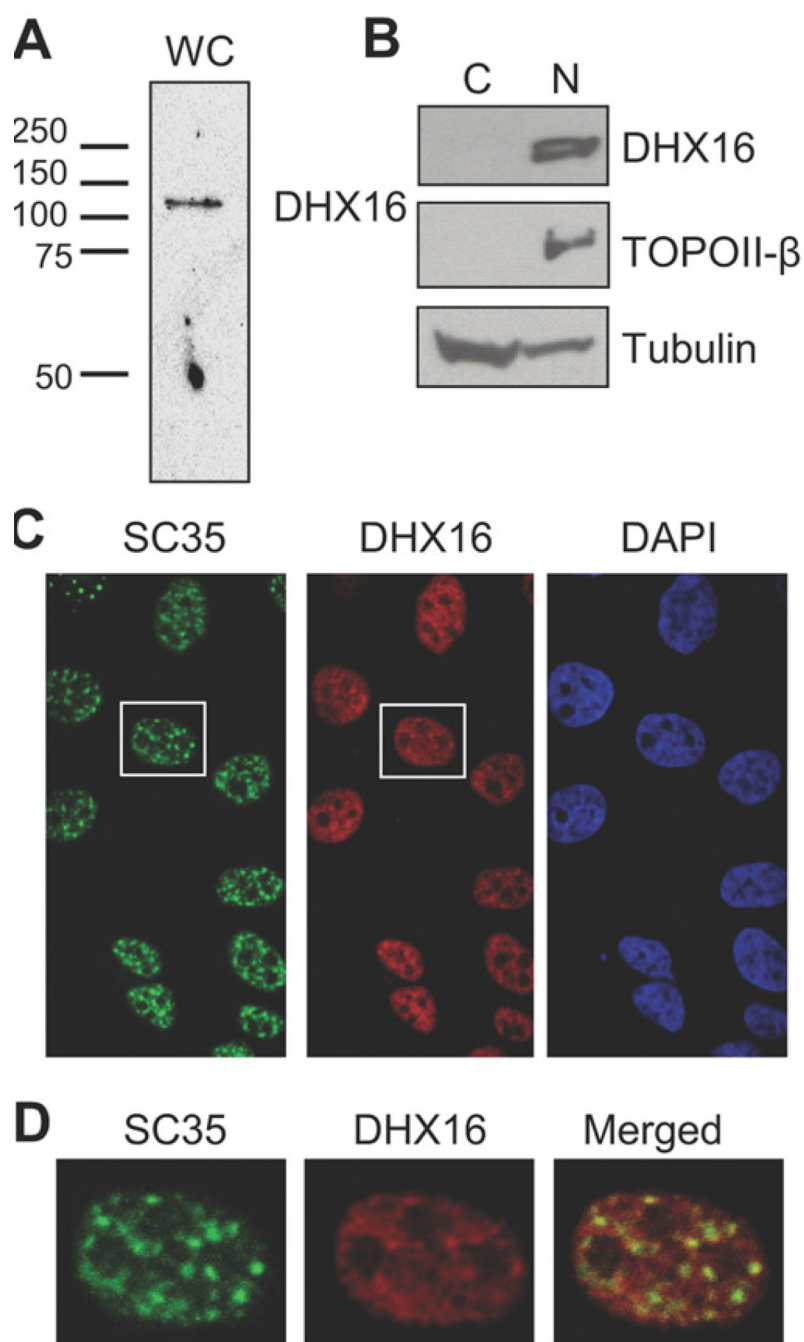


Figure 1. Cellular localization of DHX16

(A) Western blot analysis of HEK-293 whole-cell lysates (WC) using a peptide-purified antibody against a C-terminal peptide of DHX16. Positions of molecular mass markers in kDa are indicated. (B) HeLa cells were fractionated into cytoplasmic (C) and nuclear (N) fractions, and a 3×10^6 cell-equivalent of each fraction was analysed by Western blotting using anti-DHX16, anti-TOPOII- β and anti-tubulin antibodies. (C) HeLa cells immunostained with anti-SC35 (green) and anti-DHX16 (red) antibodies, and DAPI (blue) for detection of nuclei. (D) Magnified images of the cell outlined by a white rectangle in (C), shown with anti-SC35 antibody staining (green), anti-DHX16 antibody staining (red) and the merged image.

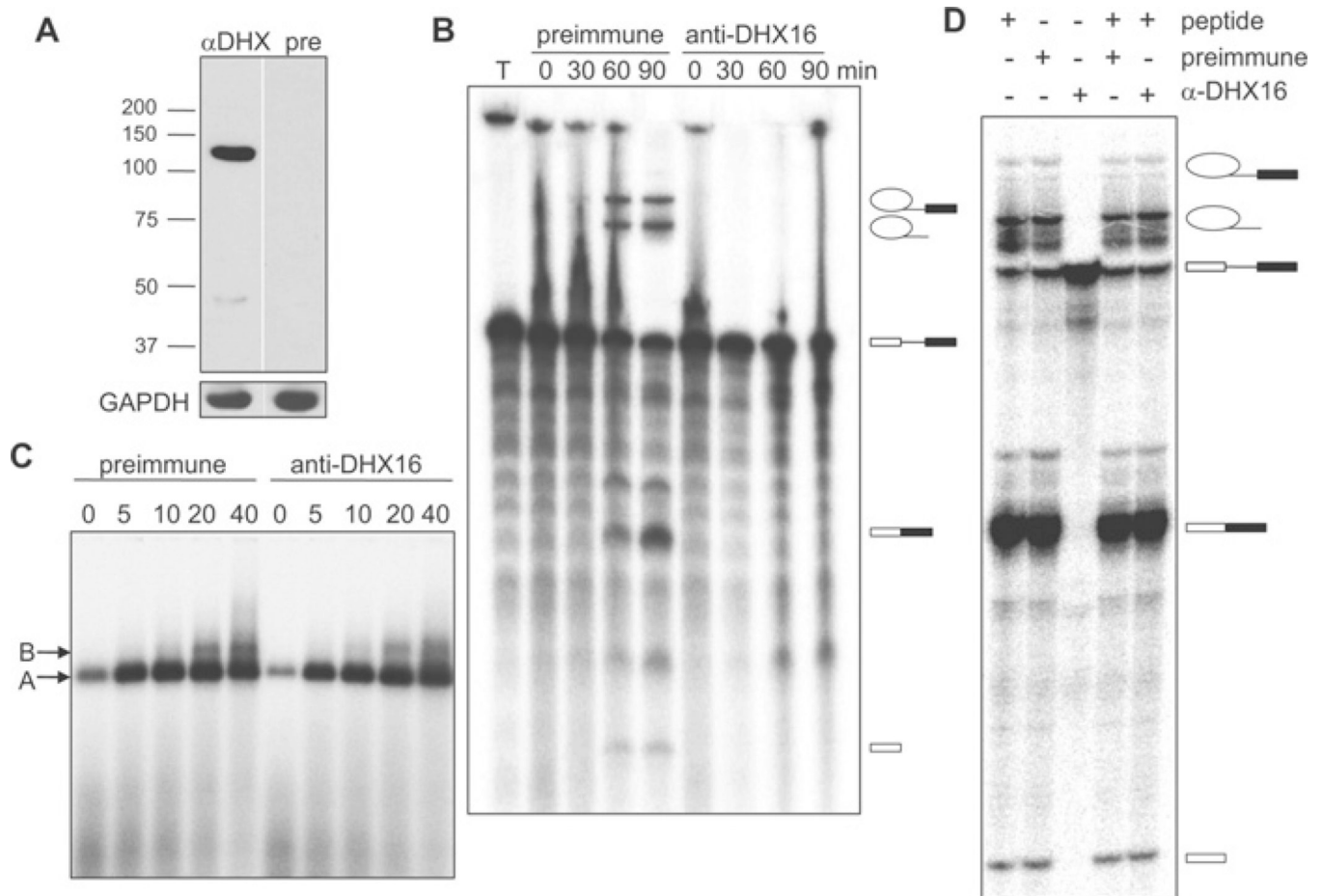


Figure 2. Anti-DHX16 antibody inhibits splicing but not spliceosome formation *in vitro*
(A) Western blot of HeLa nuclear extract with anti-DHX16 antiserum (α DHX) or pre-immune serum (pre). The lower panel shows the same blot probed with an anti-GAPDH antibody as a loading control. Positions of molecular mass markers in kDa are indicated. **(B)** Time course of *in vitro* splicing of pre-mRNA from pRG1 incubated with HeLa nuclear extracts in the presence of pre-immune or anti-DHX16 antiserum. Lane 'T' shows the pre-mRNA transcript only (i.e. not incubated with the extract). RNA molecules are indicated to the right of the gel and are, from top to bottom, IVS (intervening sequence or intron)-exon 2, intron, pre-mRNA substrate, spliced exons and exon 1. **(C)** Native agarose gel electrophoresis of aliquots of the reactions described in **(B)**. Splicing complexes A and B are indicated. **(D)** Denaturing gel electrophoresis of RNA from the splicing reactions with HeLa nuclear extracts pre-incubated with anti-DHX16 antiserum, pre-immune serum or peptide as indicated. The splicing reactions were carried out with pRG1 pre-mRNA for 60 min. RNA molecules are indicated as in **(B)**.

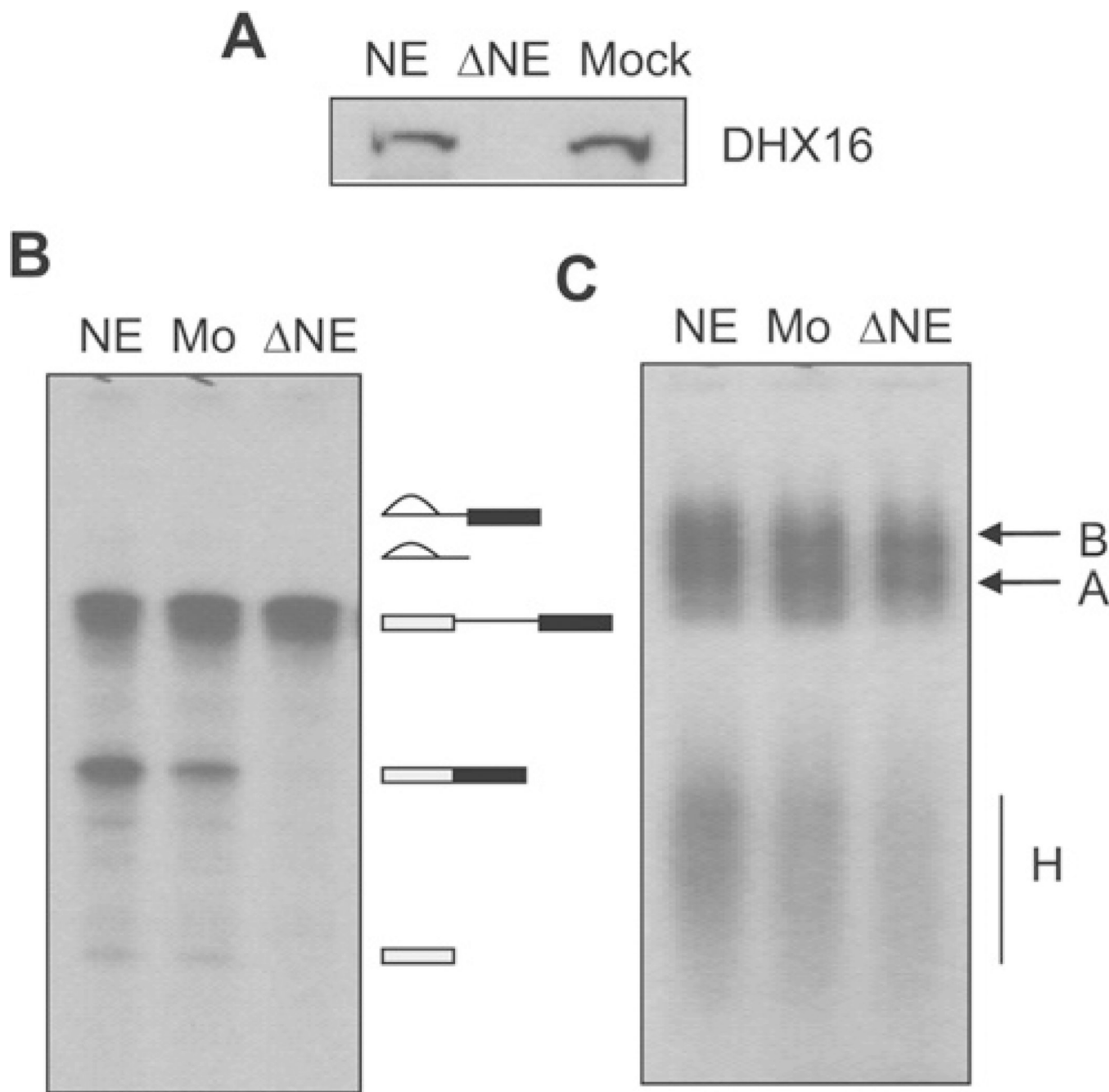


Figure 3. HeLa nuclear extracts immunodepleted of DHX16 assemble spliceosomal complex B but are not active for splicing

DHX16 was depleted from HeLa nuclear extracts with the anti-DHX16 antibody or mock-depleted with Protein A beads. NE, nuclear extract; Δ NE, DHX16-depleted NE; Mock or Mo, mock-depleted NE. (A) The degree of DHX16 depletion was verified by immunoblot analysis using the anti-DHX16 antibody. (B) Denaturing gel electrophoresis of RNA from the splicing reactions containing the indicated extracts carried out for 60 min. RNA molecules are indicated to the right of the blot and are described as for Figure 2(B). (C) Native agarose gel electrophoresis of spliceosomal complexes in an aliquot of the reactions

in **(B)** treated with 0.65 $\mu\text{g}/\mu\text{l}$ heparin. B, splicing complex B; A, splicing complex A; H, hnRNP complexes.

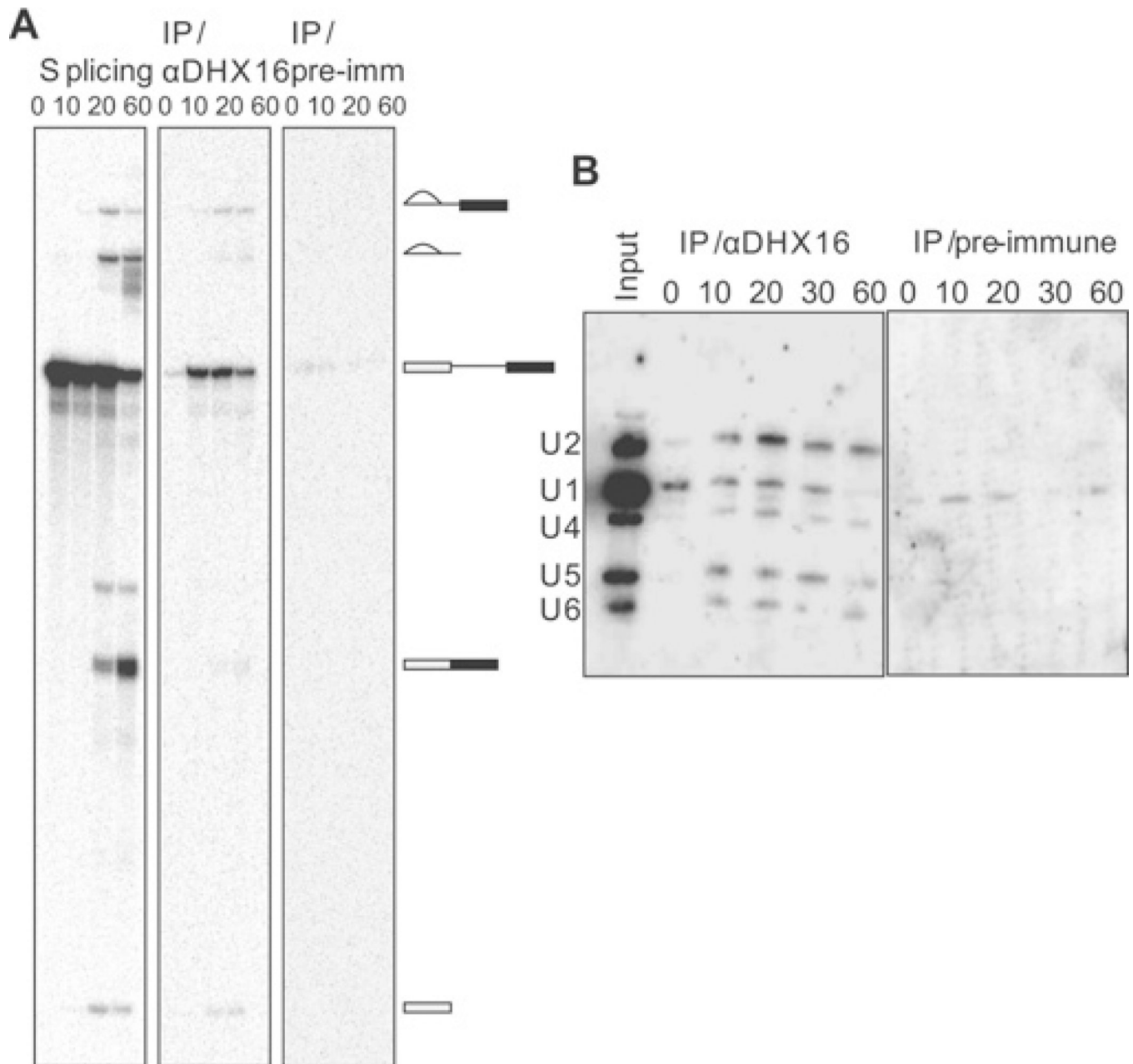


Figure 4. Pre-mRNA, splicing intermediates and snRNAs co-immunoprecipitate with DHX16
 Splicing reactions with HeLa nuclear extracts were incubated for the indicated times and immunoprecipitated (IP) with the peptide-purified anti-DHX16 antibody. **(A)** The left-hand panel shows the splicing reactions with ^{32}P -labelled pre-mRNA (no immunoprecipitation); RNA isolated from 1/15 of a 25 μl splicing reaction was applied in each lane. The middle (α -DHX16) and right-hand panel (pre-immune serum) show ^{32}P -labelled RNA that co-immunoprecipitated with DHX16; 2/3 of the RNA extracted from the immunoprecipitate of a 25 μl splicing reaction was applied in each lane. **(B)** Northern blot analyses of snRNAs co-immunoprecipitated with DHX16. Splicing reaction mixtures were incubated for the specified time and subjected to immunoprecipitation with the anti-DHX16 antibody (α -DHX16) or pre-immune serum. One-third of the RNA extracted from the immunoprecipitation reaction was run on denaturing gels, blotted and probed with ^{32}P -

labelled antisense snRNAs. Input RNA (2/15 of the RNA from the splicing reaction) was also analysed for comparison. The positions of U-snRNAs are indicated to the left of the blot.

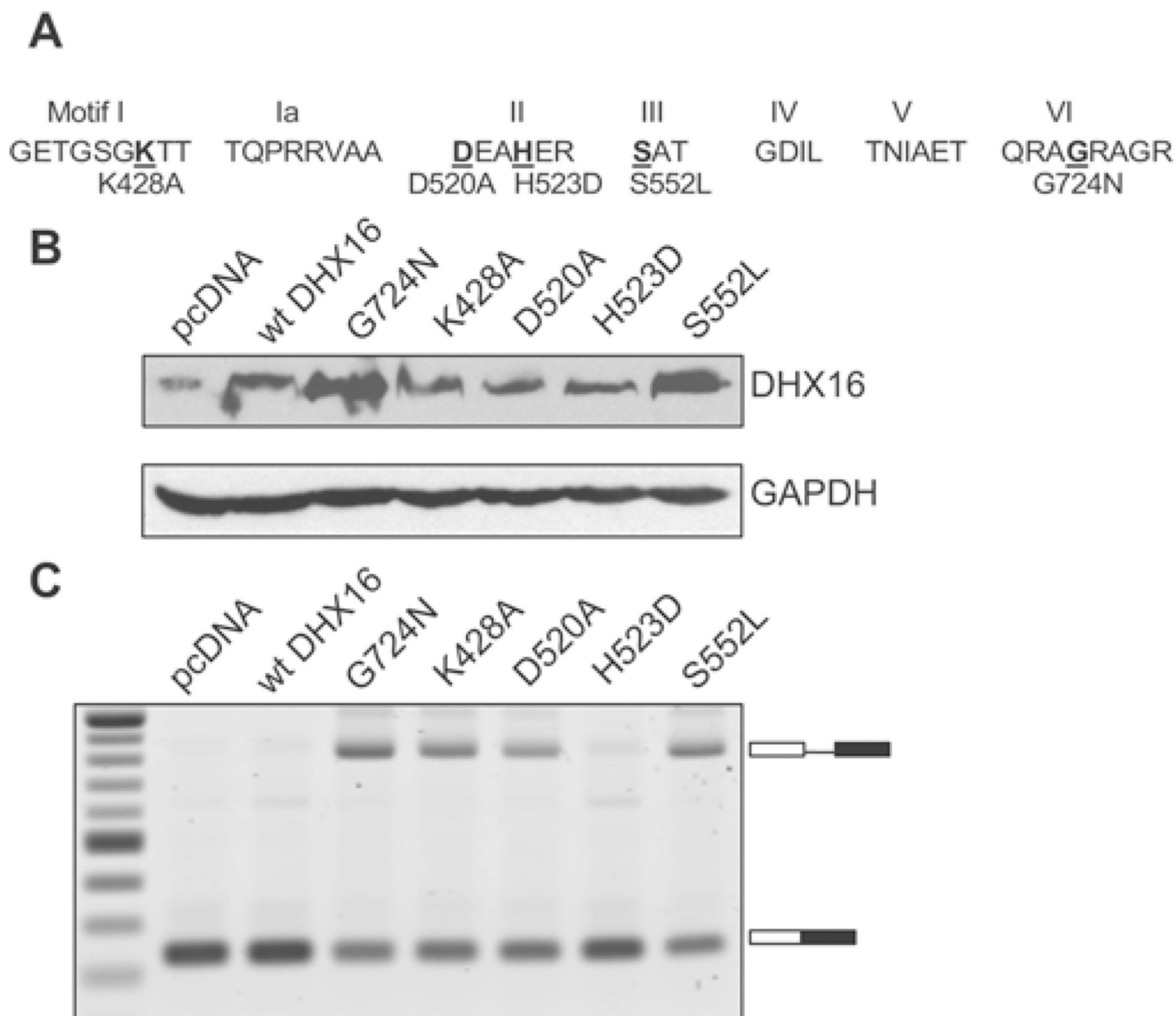


Figure 5. DHX16 mutants block splicing of a minigene transcript *in vivo*

HEK-293 cells were transfected with the pL53In minigene together with a DHX16 plasmid as indicated. (A) Schematic representation of the DHX16 mutants. Seven conserved motifs (I to VI) in the helicase domain are depicted. The mutated amino acid is shown in bold and underlined; the substituted amino acid is shown below with its protein sequence position. (B) Western blot using the anti-DHX16 antibody or anti-GAPDH antibody (control) on protein lysates of cells transfected with the mutant DHX16. (C) RT-PCR with total RNA isolated from the transfected cells. The unsliced and spliced RNAs are indicated on the right (box, exon; line, intron).

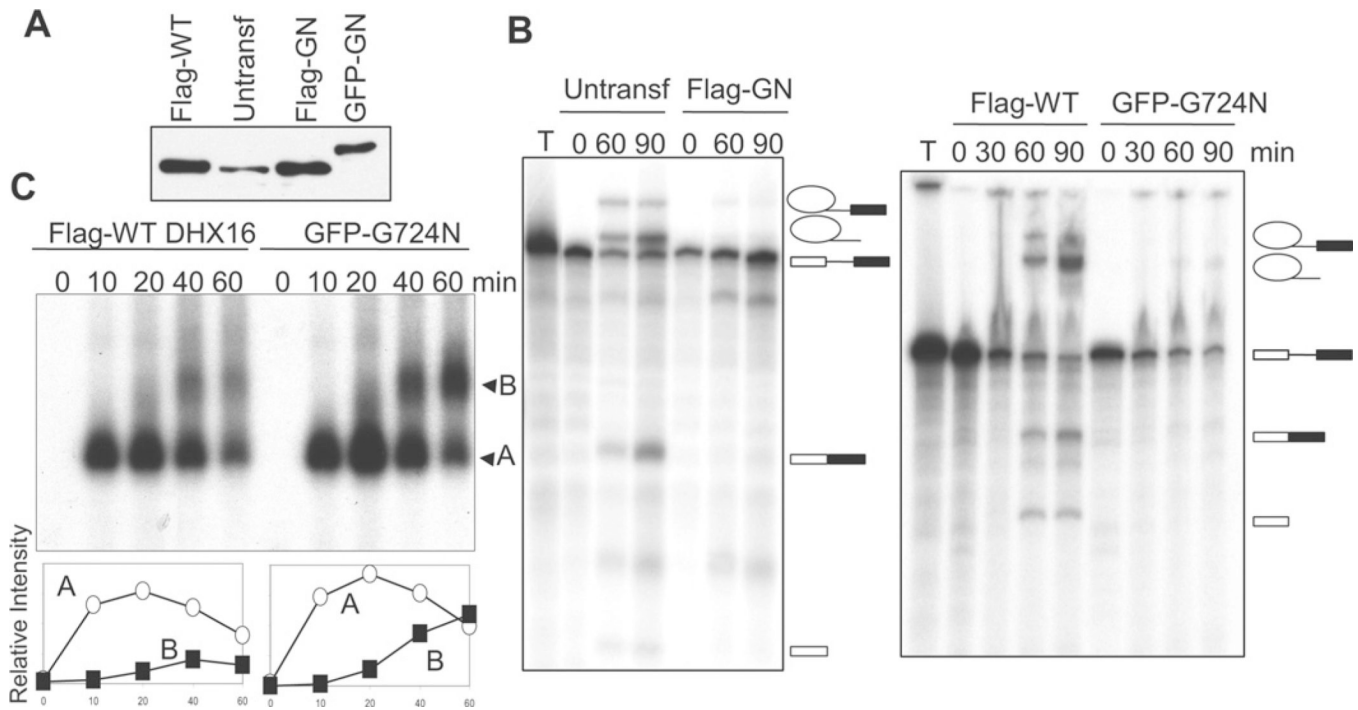


Figure 6. Nuclear extracts from mutant DHX16-expressing cells assemble spliceosomal complex B, but are defective in splicing *in vitro*

Nuclear extracts were isolated from exponentially growing, untransfected HEK-293 cells (untransf), as well as from HEK-293 cells transfected for 72 h with FLAG-tagged wild-type DHX16 (Flag-WT), FLAG-tagged mutant DHX16-G724N (Flag-GN) or EGFP-tagged mutant DHX16-G724N (GFP-GN). (A) Western blot of equal volumes of the extracts probed with the anti-DHX16 antibody. (B) Splicing reactions were carried out with the indicated extracts and for the indicated times and the RNAs from the reactions were resolved on a polyacrylamide/urea gel. T, pre-mRNA substrate that was not incubated with extracts. Pre-mRNA substrate from pRG1 was used for the reactions shown in the left-hand panel and pre-mRNA from AdML was used for those in the right-hand panel. RNA molecules are indicated to the right of the blot and are as described for Figure 2(B). (C) Splicing reactions were carried out using AdML pre-mRNA substrate in extracts from FLAG-tagged wild-type-DHX16- or EGFP-tagged mutant-DHX16-G724N-expressing cells for the indicated times and resolved on a non-denaturing gel. Spliceosomal complexes A and B are indicated. The graphs below the autoradiograph show the amount of spliceosome complex at each time point; complex A (○); complex B (■).