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Characterization of a human SWI2/SNF2 like protein hINO80: Demonstration of catalytic and DNA binding activity

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Abstract

The proteins belonging to SWI2/SNF2 family of DNA dependent ATPases are important members of the chromatin remodeling complexes that are implicated in epigenetic control of gene expression. We have identified a human gene with a putative DNA binding domain, which belongs to the INO80 subfamily of SWI2/SNF2 proteins. Here we report the cloning, expression, and functional activity of the domains from h*INO80* gene both in terms of the DNA dependent ATPase as well as DNA binding activity. A differential expression of the various domains within this gene is detected in human tissues while a ubiquitous expression is detected in mice. The intranuclear localization is demonstrated using antibodies directed against the DBINO domain of hINO80.
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Keywords: Ino80; SWI2/SNF2; Chromatin remodeling; DNA binding domain; DNA dependent ATPase

The compaction of eukaryotic genome into chromatin structure has profound implications on nuclear processes such as replication [1,2], transcription [3,4], DNA repair [5], and recombination [6,7]. Dynamic changes in chromatin structure underlie much of the transcriptional control in the eukaryotic nucleus. These structural changes are largely mediated by covalent modifications of the flexible N-terminal amino acids of the core histones by enzymes. Potential modifications include histone acetylation, methylation, phosphorylation, ubiquitylation, and ADP-ribosylation [8–10]. A second line of chromatin alterations is brought about in more subtle and transient ways. These so-called chromatin remodeling factors alter histone–DNA interactions such that nucleosomal DNA becomes much more accessible to interacting proteins. These perturbations of the nucleosome may lead to the relocation of histone octamers from a particular DNA fragment to the available acceptor DNA in *cis* or *trans*, establishing a "fluid" state of chromatin in which the overall packaging of DNA is maintained, but individual sequences are transiently exposed to interacting factors [11–15].

A distinguishing hallmark of this latter type of nucleosome remodeling is a dedicated ATPase subunit of the SWI2/SNF2 family of ATPases. The enzymes in this family can be grouped into several subfamilies according to sequence features outside of their ATPase domains [16,17]. Some of the well-characterized subfamilies of SWI2/SNF2 family of ATPases include the SNF2 subfamily (Snf2, Sth1, hBRM, and BRG1), ISWI subfamily (Iswi1, Iswi2, SNF2l, and SNF2h), CHD1 subfamily (CHD1, Mi-2 α/CHD3, and Mi2 β/CHD4), and the RAD54 subfamily (Rad54, ATRX, and ARIP4) [17]. SWI2/SNF2-like proteins participate in various nuclear activities including transcriptional control [18,19], DNA repair, chromosome segregation [20], and chromosome folding [21]. The members of two SWI2/SNF2-type subfamilies, ATRX and DDM1, are involved in the control of DNA methylation [22,23].

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The INO80 complex in yeast is the recently described chromatin remodeling complex comprising of 12-subunits [24]. The INO80 complex remodels chromatin, facilitates transcription in vitro, and displays 3′–5′ DNA helicase activity and in yeast it is known to be associated with chromosomes of dividing cells. Based on sequence analysis and domain architecture among the SNF2 like ATPases, we reported the recognition of a new subfamily of SWI2/SNF2 chromatin remodeling proteins, designated INO80 subfamily [25]. Further we identified a novel putative DNA binding domain (DBINO) in all members of the INO80 subfamily [25]. In the present study, we report the functional characterization of the human *IN080* gene product designated as hINO80. We describe the nuclear localization of hINO80 protein and the expression pattern of the *INO80* gene in the human and mouse tissues.

Materials and methods

Plasmid expression constructs. The 5979 bp cDNA clone of h*INO80* gene (GenBank Accession No. NM_017553) was obtained from Kazusa cDNA Research Institute, Japan. The region spanning the helicase domain (phINO80$_{1700–3900}$) was PCR amplified using primers R 5′-TTTGGGGAGGAATCCAGCCTGGCTAACCCA-3′ and S 5′-ACCT GAAATAAGCTTCCGCTGAATCTCACT-3′. The resultant 2.2 kb amplicon was digested with *Bam*H1 and *Hin*dIII, and directionally subcloned into pET 28(a) expression vector (Novagen) with a 6× His tag at the N and the C terminus. The recombinant plasmid was named pHel. The 800 bp region spanning the DBINO domain was also PCR amplified using the primers DB-1 5′-CTGCTCTCCGGATCCACCCTTTCTATGAG-3′ and DB-2 5′-ATTTTCAGCACTCGAGGGCCTGGGC-3′. The amplicon was digested with *Bam*HI and *Xho*I, and directionally subcloned into pGEX-5X3 expression vector (Amersham Biosciences) with a GST tag at the N terminus. This recombinant construct was named pDBINO. Both the constructs were sequenced to ensure the correct reading frame and the sequence.

Recombinant protein expression. The protein expression was performed in BL21-RIL CodonPlus *Escherichia coli* cells (Staratagene) in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol for pDBINO and 25 μg/ml kanamycin and 34 μg/ml chloramphenicol for pHel carrying recombinants. Cultures were grown to a density of 0.6 A_{660}, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM and induced for 4 h at 37 °C, before cells were collected by centrifugation at 10,000*g* for 20 min. Cell pellets were resuspended in the sonication buffer {50 mM Tris–HCl (pH 7.5), 200 mM NaCl, and 1 mM PMSF} and sonicated with six bursts of 15 s at 4 °C, using the Sonopuls Ultrasonic homogenizer (Bandelin Electronic, GmbH). The insoluble material was removed by centrifugation at 4 °C at 10,000*g* for 30 min.

Purification of 6× His tagged protein was carried out using Ni–NTA resin (Bangalore Genei) as described previously [26]. The bound recombinant protein was eluted with a step gradient of 20–500 mM imidazole in buffer A containing 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, and 10 mM imidazole. Purification of GST tagged protein was carried out using GST–Agarose (SigmaChemical) as per manufacturer's recommendation. The protein was eluted with 0.15 mM reduced glutathione.

DNA-dependent ATPase assay. DNA-dependent ATPase activity was determined with a colorimetric assay measuring the formation of inorganic phosphate as previously reported [27,28]. Standard reaction mixture (50 μl) contained 20 mM Hepes (pH 7.0), 5% glycerol, 50 mM potassium acetate, 8 mM $MgCl_2$, 1 mM ATP, and 20 ng/μl of supercoiled plasmid pSK+ (Stratagene) DNA or mononucleosomal fraction from nuclei isolated from human placenta. Protein sample was added to the reaction mix and incubated at 30 °C for 30 min. Reaction was terminated by the addition of 0.85 ml malachite green/acid molybdate solution followed by addition of 0.1 ml of 34% (w/v) sodium citrate solution. After 15 min of color development, A_{630} was determined.

Electrophoretic mobility shift assay. EMSA was performed as described previously [29], in a 30-μl volume in the DNA binding buffer: 20 mM Hepes, pH 8.4, 100 mM KCl, 0.12 mM EDTA, 6 mM β-mercaptoethanol, 20 mM dithiothreitol, and 120 μg/ml BSA, in the presence of end-labeled DNA fragment. The samples were electrophoresed on a pre-run 6% native acrylamide–bisacrylamide (28:2) gel in 1× Tris–borate–EDTA at 4 °C. The gel was dried and subjected to autoradiography. Binding was tested with two templates having random sequences, one a 300-bp fragment from *FMR1* gene and another a pool of double stranded oligo nucleotides of 56 bp length with random sequence.

Fluorescence spectroscopic analysis. Steady-state fluorescence emission intensity measurements were performed with a Spex Fluoromax-3 spectrofluorimeter. Excitation and emission slits with a nominal band-pass of 5 nm were used for all measurements. Background intensities of samples without protein were subtracted from each spectrum to avoid any contribution from solvent, Raman peak, and other scattering artifacts. Intrinsic fluorescence of the protein was used to analyze DNA–protein interactions. Fluorescence titration was performed using fixed concentration of DBINO-GST fusion protein (2.5 μg) and varying concentrations of DNA (150–1300 ng). All buffers used were filtered through 0.2 μm filters. The mixtures were stirred continuously in a 1.0-cm path length cuvette to ensure homogeneity and all titrations were carried out at 20 °C. Protein sample was excited at 241 nm in Hepes buffer. Tryptophan residues in the protein would get excited at this wavelength. The emission was measured at 300–450 nm wavelength range. Further aliquot of DNA was added until there was no further change in fluorescence intensity in emission spectrum. The change in fluorescence intensity at 341 nm was then used to interpret the data. Similar steps were performed in buffers of different salt concentrations (0–150 mM NaCl).

Expression analysis. Human MTC Panel 1 (BD Biosciences Clontech) containing first strand total cDNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was used to check the expression of h*INO80* gene. Three different sets of primers spanning different exons were used. (EX-1: TGG ACC ATT TTC TGC GAC AA; EX-2: GAT GGC GAC GAG GGG ATT CTT C; ATP-1: GAT GAA ATG GGC CTT GGT AAA ACA; ATP-2: TAG CAA AAG CCG ATT CCG ACA CTG; LE-1: TGTTCCCAGCAATTCCTCC; LE-2: ATTCCCCTATACCCCC TACTCC). For expression analysis of *Ino80* gene in mice, total RNA from brain, heart, kidney, lung, skeletal muscle, liver, spleen, and testis was isolated using EZ-RNA isolation kit (Biological Industries, Israel) following the manufacturer's protocol. Similar protocol was applied for the extraction of total RNA from human embryonic kidney (HEK) cells also. The quality of RNA was analyzed by electrophoresis on 1.5% agarose gel. One microgram of total RNA was used for cDNA synthesis using PowerScript Reverse transcriptase (Clontech). The presence of cDNA for the ubiquitously expressed β-globin gene by PCR amplification with gene specific primers served as a control for the quality of cDNA. The PCR primers were designed such that they map within exons and the amplicon of the expected sizes would be obtained only if the cDNA was used as the template. Three sets of primers were used for mouse *Ino80* gene spanning different exons (MSH-1: TACCAAGACCCATTTCATCAGC; MSH-2: AGTAACCATTTTAAAGCCCAGG; MLE-1: CTGTAAGGATGAGA GGCTGGAG; MLE-2: CTAAACTCAATTCCGCGATTTC; MEX-1: AGACGTGATGAAGAGTTTTCC; MEX-2: CTTCCTGGATTCCATC ATGAC).

Immunoblotting and immunofluorescence. The hINO80 protein sequence was analyzed using the GCG Wisconsin package and two peptide corresponding to amino acids 195–209 (DPFYEQQRHLLGPKK), designated RB1, and 1310–1324 (KEDELDGKRRKEGVN), designated RB2, were synthesized for generation of polyclonal antibodies. The polyclonal antibodies against RB1 and RB2 were raised in rabbit and mouse, respectively, and were used for the detection of recombinant DBINO and in situ immunofluorescence.

The total cell lysate from human embryonic kidney cell line was prepared and used for immunoprecipitation experiments. After preclearing with protein A–agarose and incubation with AntiRB1 antibodies, the

complex was pulled down with protein A–agarose. The proteins in the complex were released from the beads and analyzed by electrophoresis on 10% SDS–PAGE. For immunofluorescence staining, HEK cells were grown on collagen-coated coverslips in RPMI medium, washed briefly in PBS (pH 7.1) and permeablized in 0.1% (v/v) Triton X-100 in PBS for 10 min and fixed in paraformaldehyde [3% (v/v)] for 15 min and washed in PBS. They were incubated in 1% BSA and 0.5% Triton-X for 45 min, before incubation with the primary antibody for 2 h at room temperature. After washing three times in PBS, they were treated with secondary antibody in 1% BSA for 1 h at room temperature and washed three times in PBS before mounting. The cells were counterstained with DMA an analogue of Hoechst 33258 [30]. The slides were observed under 100× using Nikon Fluorescence Microscope, Eclipse E600W.

Results

Identification of hINO80: a novel member of SNF2 family

In silico analysis led to the identification of several members of a new subfamily of SWI2/SNF2 superfamily across phyla, namely the INO80 subfamily [25]. One of the members of this subfamily is a previously uncharacterized protein hINO80 comprising of 1556 amino acids with a theoretical p*I* of 9.53 and a predicted molecular mass of 177 kDa. The h*INO80* gene comprises of 36 exons, which span a total chromosomal region of 137 kb on 15q15.1. The homologue of the hINO80 was also identified in mouse (GenBank Accession No. XP_355376.1).

The hINO80 protein contains the characteristic seven motifs of the SNF2 helicase domain that are dispersed over a region of 700 amino acids (Fig. 1A) and the putative DNA binding domain DBINO that we identified previously. hINO80 exhibits a strong nuclear localization signal (KKLKAKLKKVKKKRRR) at N-terminal as well as (RKRKREKYAEKK) at the C-terminal of the protein (Fig. 1A). To experimentally validate the predicted functions of the hINO80 protein, we expressed the two highly conserved regions of the protein, one spanning the SNF2 ATPase domain and other the DBINO domain as recombinant proteins with 6× His tag and GST tag, respectively (Figs. 1B and C).

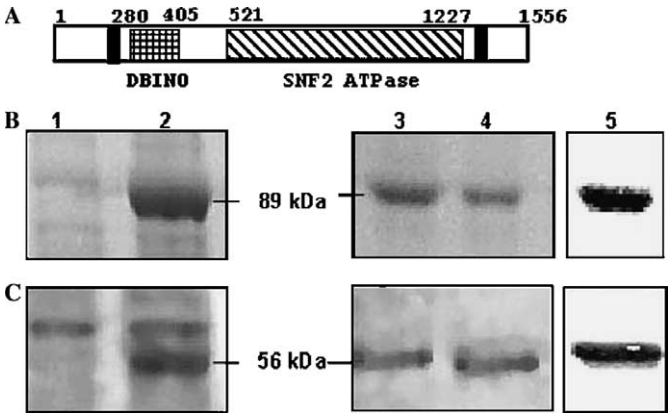

Fig. 1. Expression of conserved domains of hINO80 as fusion proteins. (A) Line diagram showing the relative positions of the putative DNA binding domain (DBINO) and the SNF2 ATPase domain in the SNF2-R protein, NLS-nuclear localization signal. (B) Expression of the cloned SNF2 ATPase domain and (C) expression of the DBINO domain in BL21 cells. Lanes 1 and 2 represent the Coomasie blue stained fusion protein analyzed by electrophoresis from uninduced and induced recombinant *E. coli* cells, respectively, lanes 3 and 4, fusion proteins purified by affinity chromatography; lane 5, detection of fusion proteins; SNF2 ATPase and DBINO domain with antibodies against His- and GST tag, respectively, by Western analysis.

hINO80 has a DNA dependent ATPase activity

The recombinant protein containing SNF2 ATPase domain of hINO80 protein was purified and assayed for ATPase activity under various conditions. The protein showed ATPase activity only in the presence of double stranded DNA and negligible activity was detected in the presence of single stranded DNA or RNA (Fig. 2). No ATPase activity was detected in the absence of DNA. In the presence of mononucleosomes isolated from human placenta, the activity was similar to that with double stranded DNA. The fractions consisting of potential contaminating proteins from extracts of BL21 (DE3) bacterial cells harboring the pET 28a vector failed to show ATPase activity with and without DNA. Kinetic analysis of the hINO80 SNF2 domain was carried out by varying the ATP concentration (100 μM–2 mM) in the presence of DNA. A time course of ATP hydrolysis in presence of dsDNA by hINO80 ATPase established that the reaction was linear for 60 min and therefore for further analysis ATPase reaction was carried out for 30 min. The Michaelis–Menten constant (K_m) was then determined by double-reciprocal plot and compared with those of other remodeling factors. The Fig. 3D shows the Lineweaver–Burk plot for the ATP hydrolysis by the hINO80 ATPase. The K_m as calculated is 167 μM. This was found to be similar to that of other SNF2 family ATPases reported, which varies from 100 to 550 μM [31]. The hINO80 ATPase was assayed for the optimum temperature, pH, and concentration of divalent cations (Figs. 3A–C). The DNA dependent ATPase assays were performed for 30 min in the presence of 20 ng/μl plasmid DNA and 1 mM ATP. The assay was

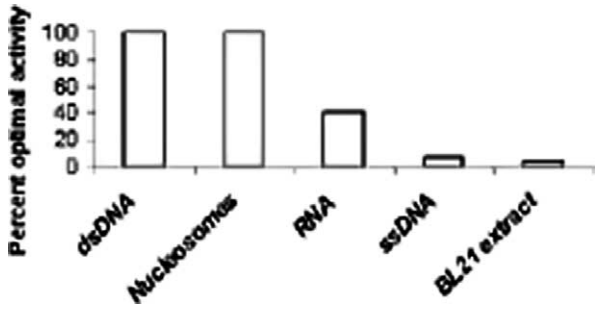

Fig. 2. Detection of ATPase activity in the cloned SNF2 ATPase domain of hINO80. The activity is expressed relative to the activity in presence of double stranded DNA taken as 100%. Double/single stranded DNA and RNA were used at a concentration of 20 ng/μl in the final reaction mix. Column 5 is the ATPase activity in BL21 (transformed with pET28a vector) extract in presence of double stranded DNA.

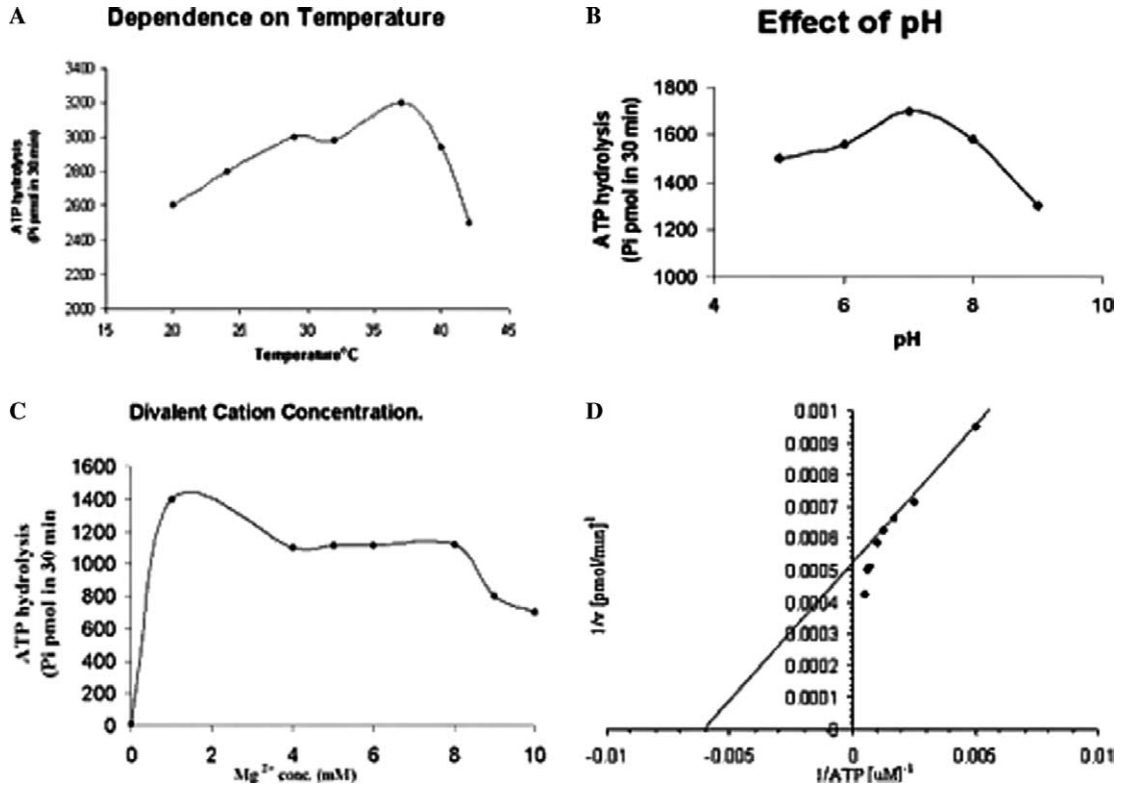

Fig. 3. Analysis of the catalytic activity of the recombinant, SNF2 domain of hINO80 protein. (A–C) Dependence of ATPase activity on temperature, pH and divalent cation, (D) double reciprocal plot for kinetic analysis where assays were performed with increasing concentration of ATP (100 μM–2 mM) in presence of constant amount of double stranded DNA. The double reciprocal Lineweaver–Burk graph (1/reaction velocity, $1/v$ (1/pmol ATP hydrolyzed min^{-1}) versus 1/ATP) was plotted data obtained from the standard ATPase assay containing 150 ng hINO80 with varying concentrations of ATP after incubation for 30 min.

carried out in the temperature range 20–45 °C. The optimum temperature for ATPase activity was 37 °C. Maximal activity was observed at pH 7.0. The optimum divalent cation concentration was 5–8 mM.

DBINO domain has DNA binding activity

We have earlier identified a putative DNA binding domain towards the N-terminal region of ATPase domain and designated it DBINO [25]. To demonstrate the DNA binding activity of the DBINO domain, the electrophoretic mobility shift assays (EMSA) were carried out with 300 bp fragments of DNA of random sequence. The retardation in mobility was observed and the retardation increased with increasing concentration of the protein and saturated at a molar ratio of 1:9.3 (pmol/pmol, Fig. 4A). Thus suggesting that on a 300-bp fragment multiple protein molecules would bind and approximately one protein molecule appears to interact with about 30 bp DNA. This was further borne out when we used a pool of end labeled double stranded random sequence oligonucleotides of about 56 bp each (Fig. 4B). There is no alteration in the apparent size of the complex with increase in protein concentration. Thus indicating that there are perhaps only one or two protein binding sites on the template DNA. Further the possibility that the increased retardation observed with increasing concentration of the protein seen in Fig. 4A is due to aggregation is ruled out by this experiment.

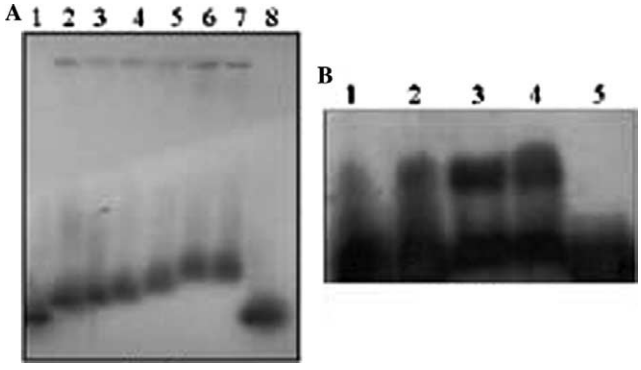

Fig. 4. Demonstration of DNA binding activity of DBINO domain of hINO80. (A) Electrophoretic mobility shift assay was carried out with a 300 bp DNA fragment end labeled with [γ-^{32P}]ATP. One picomole of DNA was used in all the lanes. Lane 1, DNA alone; lane 2–7 labeled DNA with the fusion protein at various concentrations; lane 2, 0.1 pmol; lane 3, 2.5 pmol; lane 4, 4.6 pmol; lane 5, 6.9 pmol; lane 6, 9.28 pmol; and lane 7, 11.6 pmol. Lane 8, DNA with GST protein as a negative control. (B) Demonstration of the absence of oligomerisation of DBINO. EMSA was carried out with short double stranded oligonucleotides of 56 bp end labeled with [γ-^{32}P]ATP. One picamole of DNA was used in all the assays, protein concentration was varied, lane 1, 5 pmol; lane 2, 15 pmol; lane 3, 25 pmol; lane 4, 50 pmol; and lane 5, oligonucleotide alone without protein.

Spectroscopic analysis

Fluorescence emission spectra of recombinant DBINO in the absence and presence of different amounts of DNA were recorded in order to study the binding event. The recombinant protein has five tryptophan residues, two coming from the DBINO domain and three from GST. Fig. 5A shows emission spectra of recombinant DBINO on addition of DNA. It is seen that addition of increasing concentrations of DNA results in a gradual increase in the fluorescence intensity of recombinant DBINO. In case of GST alone, such titration does not show any change in the fluorescence intensity of GST (Fig. 5B). These spectral changes in the emission spectra of recombinant DBINO arise from the change in microenvironment of the protein revealing that recombinant DBINO is interacting with DNA. To determine whether the interaction between recombinant DBINO and DNA is specific or non-specific (electrostatic), similar titration was also performed in the presence of 150 mM NaCl. It is expected that in the presence of high concentration of salt, ions in the buffer will shield electrostatic charges of the macromolecules and thus lower the repulsion between DNA and recombinant DBINO, if any, and will prevent non-specific protein–DNA interactions. Ratio of the fluorescence intensity of recombinant DBINO in the presence of DNA (I) to that in the absence of DNA (I_0) was plotted as a function of DNA concentration (Fig. 5C). The I/I_0 values at the saturation point were around 4 and 3 in the absence and presence of NaCl, respectively. Substantial value of I/I_0 in presence of high salt concentration, which is marginally lower than that in the absence of salt, indicates that the binding event is composed of two kinds of interactions, specific and non-specific. Lower slope for the first few points (before saturation) in the plot of I/I_0 vs. DNA concentration in presence of NaCl compared with that in the absence of NaCl suggests the suppression of non-specific interaction in the presence of salt.

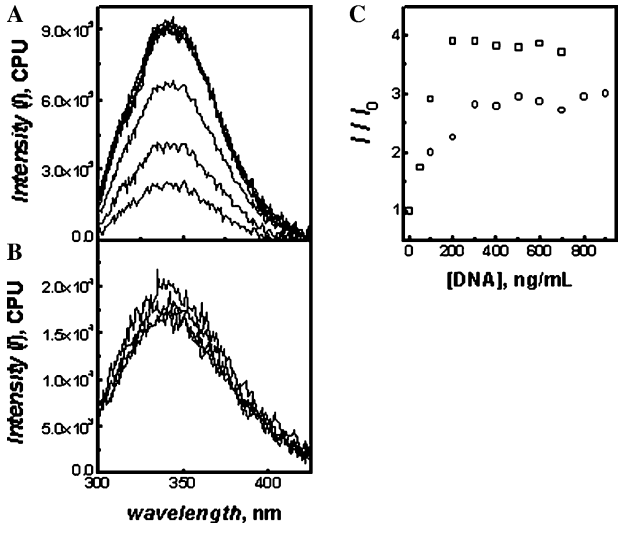

Fig. 5. Analysis of DNA binding by fluorescence spectroscopy. DBINO (A) and GST (B) in absence and presence of different amounts of DNA. Spectra were recorded at 20 °C in Hepes buffer, pH 7.0. (C) Variation of I_0/I as a function of DNA concentration, where I_0 and I are the fluorescence intensities of the protein at 340 nm in absence and presence of DNA, respectively, □ in absence of NaCl, -○- in presence of 150 mM NaCl. Protein concentration was 0.1 mg/ml in both the cases.

Expression pattern of hINO80 gene

Many of the *SNF2* genes show ubiquitous patterns of expression suggesting their involvement in normal cellular functions and the regulation of housekeeping genes. We carried out expression analysis of the *INO80* gene in mouse and humans to study its tissue expression patterns. Three primer sets mapping across different exons and away from splice junctions were used for PCR on cDNA samples (Fig. 6A) and all the three showed expression in all the tissues from mouse that we examined (Fig. 6). But in cDNA from human tissues two of the primers show ubiquitous expression but the transcript, which spans the SNF2 ATPase domain, is expressed only in brain, liver, and pancreas in humans. PCR with control primers that amplify a fragment of *G3PDH* gene showed expression in all the tissues confirming the quality of the cDNA samples (data not shown). Moreover, presence of specific amplicon with two sets of primers in all tissue cDNA samples confirmed the quality of the cDNA panel.

Expression analysis of hINO80 in HEK cell line

Human embryonic kidney (HEK) cells were used for the analysis of the intracellular localization of hINO80. The h*INO80* gene transcript was detected in HEK cells by RT-PCR with specific primers mapping to the ATPase and the last exon of h*INO80* gene (Fig. 7). This suggests that in the embryonic kidney cell line the h*INO80* transcript containing the SNF2 helicase domain is expressed. The expression of hINO80 protein was also assayed by immunoprecipitation followed by Western blotting. The nuclear extract from HEK cells was immunoprecipitated with anti-RB1 antibodies and fractionated by electrophoresis on denaturing polyacrylamide gel (Fig. 7C). A strong band was detected at 76 kDa range. To ensure the detection of hINO80, immunoprecipitated fraction as well as the cell lysate from HEK cells was subjected to Western blot analysis with antibodies raised against another peptide specific to hINO80, namely the RB2. The anti-RB2 antibodies detected a band at the same molecular weight range (76 kDa) in both the total cell lysate and the immunoprecipitated complex (Fig. 7D). It is probable that this is one of the alternate splice products in HEK cells and not the full-length transcript from *INO80* gene.

Intracellular localization of the hINO80 protein

Having confirmed the expression of hINO80 in human HEK cells, the intra-cellular localization of hINO80 protein was investigated by in situ immunofluorescence

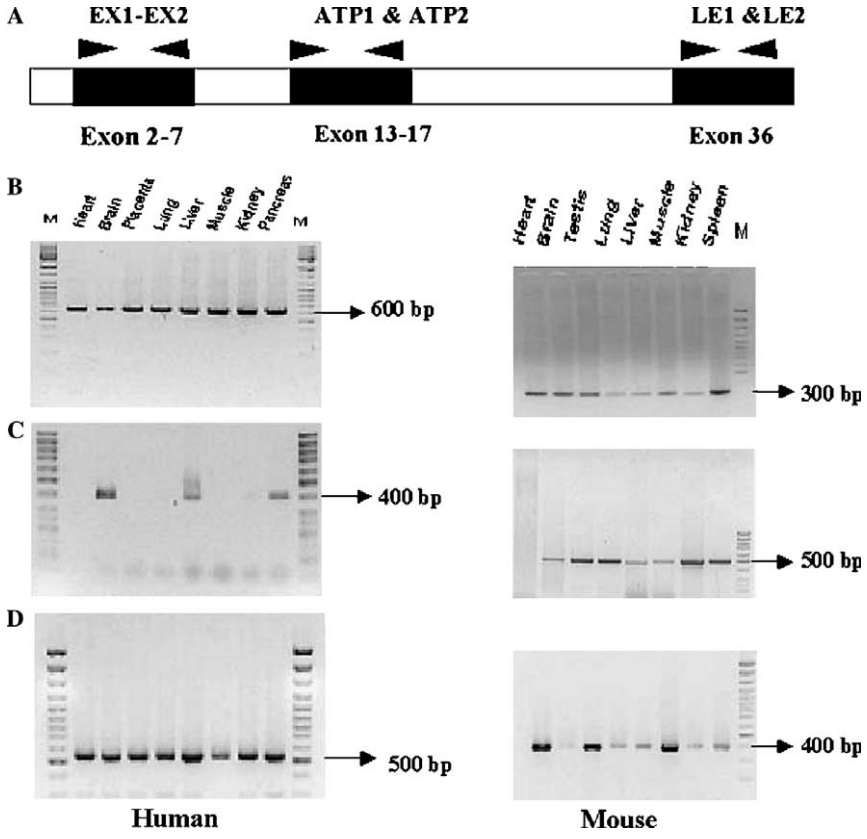

Fig. 6. Expression analysis of human and mouse *INO80* gene. Expression profile in human and mouse tissues was screened by PCR of tissue specific cDNA libraries and total tissue cDNA, respectively. (A) Diagrammatic representation of the three sets of human/mouse gene specific primers spanning different exons used in PCR; amplicons from different primer sets were analyzed, (B) primer EX/MEX 1 and 2, (C) primer ATP/MSH 1 and 2, (D) primer LE/MLE 1 and 2, M-100 bp ladder as size marker.

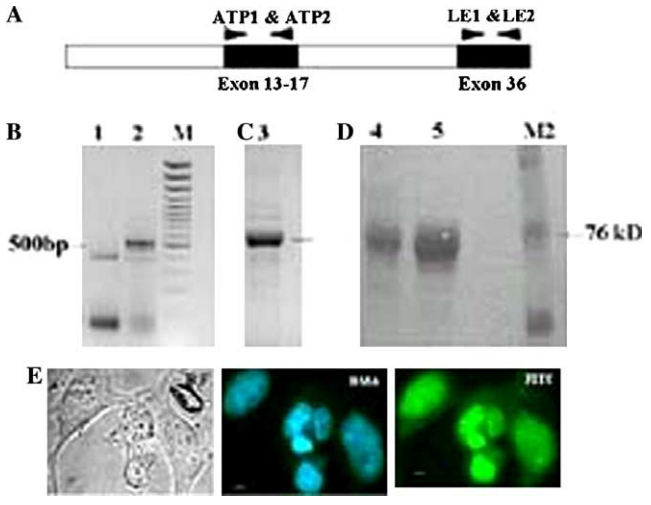

Fig. 7. Expression of hINO80 in human embryonic kidney (HEK) cells. (A) Diagrammatic representation of hINO80: arrowheads indicate primers used in RTPCR, (B) RT-PCR with RNA isolated from HEK cells, the band around 50 bp is due to primer dimers; lane 1, primers ATP 1 and 2, lane 2, primers LE1 and 2, M, 100 bp ladder as size marker. (C) (lane 3) Immunoprecipitated hINO80, (D) Western analysis with anti RB2 antibodies: lane 4, total protein from HEK cells, lane 5, protein isolated after immunoprecipitation, M2, protein molecular weight marker. (E) Intracellular localization of hINO80 protein in HEK cells. Immunostaining was done with anti RB1 antibodies.

microscopy with affinity-purified anti RB1 polyclonal antibodies (Fig. 7E). The hINO80 staining exhibited a fine granular pattern, evenly dispersed throughout the interphase nucleus, as can be seen by comparison with counterstaining with a Hoechst analogue of the same cells.

Discussion

On mining the genome for novel chromatin remodeling proteins we detected an uncharacterized putative *SNF2* like gene in human genome, mapping at 15q15.1 (GenBank Accession No. NM_017553). This putative SNF2 ATPase was initially identified as a homologue of yeast Ino80 and designated hINO80. By in silico analysis we recognized the presence of a putative DNA binding domain and extensively characterized the gene organization. The in silico predictions are validated by functional and expression analysis in the present report.

The human and mouse *INO80* genes are part of a distinct subfamily within the SNF2 family as mentioned earlier [25]. The members of the INO80 subfamily of SNF2 proteins, including hINO80, are unique in having the seven highly conserved ATPase motifs spread over 700 amino acids instead of 400 as in other SNF2 ATPases. We demonstrate the catalytic activity in terms of DNA dependent

ATPase activity of the SNF2 domain of hINO80 by expression cloning. The Km for hydrolysis of ATP in presence of DNA was determined to be 167 μM, which is similar to those of other ATPases of SWI2/SNF2 family [31]. In the context of the recently demonstrated DNA and nucleosome dependent ATPase activity of hINO80 complex, our results identify this function of the complex with the uncharacterized KIAA1259/hINO80 protein that is part of this complex [25,32].

The presence of a novel DNA binding domain, DBINO, was predicted in all the members of the INO80 subfamily by in silico analysis. Here, we demonstrate the DNA binding activity of the DBINO domain by electrophoretic mobility shift assay as well as fluorescence spectroscopy using the cloned and expressed protein. SNF2 family of DNA dependent ATPases do not exhibit sequence specificity in binding in the context of their catalytic activity. Our results indicate that the hINO80 interacts with about 30 nucleotides and may not have explicit sequence specificity. However, the results of EMSA with short double stranded oligonucleotides of 56 bp suggest that DBINO domain might confer a sequence preference, as not all the oligonucleotides seem to show retardation in mobility in spite of increased protein concentration. There is some radioactivity detected at position corresponding to the unbound oligonucleotides (Fig. 4B). The results of fluorescence spectroscopy indicate interaction between DNA and the DBINO domain. This interaction appears to have two components, one being specific and resistant to high salt and another ionic interaction sensitive to high salt concentration. This may represent ionic interaction associated with DNA dependent ATPase activity while the specific interaction may confer functional specificity to the chromatin remodeling complex where in hINO80 is one of the components.

In *Saccharomyces cerevisiae*, in a study directed at understanding the role of actin and actin related proteins (Arps) in functions of INO80.com, the authors observed that a deletion of a highly conserved region towards the N-terminal of the ATPase domain abolished DNA binding as well as Arp interaction with Ino80 in *S. cerevisiae*. The deletion included a stretch of 326 amino acids from 356 to 682 [33]. We have identified the DBINO domain of 126 amino acids overlapping this region extending from 475 to 601 amino acids in yeast. Thus, we suggest that the absence of DNA binding of Ino80 protein with N-terminal deletion is due to the loss of DBINO domain. Further, the absence of interaction of this protein with Arp 8, Arp4, and actin may be because of the loss of additional 200 amino acids deleted in Ino80 protein by [33].

Several alternate splice products are predicted in the cDNA database for *hINO80*. But the function of these transcripts is not known. In the present study, it is observed that different exons of *hINO80* exhibit variable tissue specificity in expression in human tissues. The first and the last exons are ubiquitously expressed in all the tissues studied. This suggests that the proteins encoded by the corresponding spliced RNA are perhaps involved in basic cellular mechanisms or housekeeping activities. However, the transcript containing the ATPase domain is expressed in very few tissues in humans. The exons that span the helicase domain are only expressed in brain, liver, and pancreas. Thus hINO80 seems to have evolved from being a component of a housekeeping gene regulation mechanism to that of a tissue specific gene regulation mechanism in humans. The data obtained suggest that selective expression of ATPase from *hINO80* may be regulated at mRNA splicing level rather than transcription, as the 5′ and 3′ exons are ubiquitously expressed in human tissues. This appears to be an additional regulatory mechanism in human tissues that is not present in mouse tissues, as ubiquitous expression is observed for the ATPase domain also in mice.

In *S. cerevisiae*, as mentioned earlier, the INO80 complex is known to remodel chromatin, facilitate transcription in vitro, and display 3′–5′ DNA helicase activity and in yeast it is known to be associated with chromosomes of dividing cells. In the present analysis, hINO80 was localized to the nucleus and to metaphase chromosomes in the dividing cells (data not shown). Therefore, it is possible that the INO80 protein complex might be part of the cellular memory mechanism.

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