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Characterization of human *NIPK* (*TRB3*, *SKIP3*) gene activation in stressful conditions ☆

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Abstract

The neuronal cell death-inducible putative kinase (*NIPK*) gene is upregulated in several cell types under stressful conditions. In order to understand the molecular control of the human (h) *NIPK* gene (also known as *TRB3* and *SKIP3*), we mapped the transcriptional start sites of the gene in HepG2 cells treated with thapsigargin, the inhibitor of endoplasmic reticular Ca^{2+}-ATPase, and determined the promoter region of the gene which is essential for endoplasmic reticulum and arsenite stress responses. The analysis of cDNA clones revealed the presence of several *hNIPK* mRNA isoforms, differing in their 5′ regions upstream of the hNIPK translation initiation codon as a result of alternative transcription initiation and alternative splicing. The induction of *hNIPK* gene in response to thapsigargin and arsenite treatments is mediated by a promoter segment consisting of tandemly arranged 33-bp repeats that contain a regulatory element similar to C/EBP-ATF composite site of the *Chop* gene promoter. ATF4, whose level is upregulated in the cells exposed to thapsigargin or arsenite, is able to bind to the 33-bp repeat and activate the *hNIPK* promoter. The coexpression of hNIPK inhibits activation of *hNIPK* promoter in response to the stress-inducing agents and to overexpressed ATF4, and thus NIPK may function as a negative feedback regulator of ATF4.

Keywords: NIPK; TRB3; Gene expression; Stress response; Endoplasmic reticulum; Arsenite; ATF4

In a search for genes activated in murine neuronal cell-line GT1-7 in cell death-inducing conditions, we identified *NIPK* (alias *TRB3*) as a gene upregulated during the exposure of the cells to thapsigargin (the inhibitor of endoplasmic reticular Ca^{2+}-ATPase, which evokes endoplasmic reticulum (ER) stress) and buthionine sulfoximine (the inhibitor of γ-glutamylcysteine synthetase) [1]. Mouse *NIPK* and its homologs in rat [2] and human (also known as *SKIP3* [3] alias *C20orf97* in the GenBank database) encode proteins with substantial similarity to subdomains IV–XI of a common catalytic core structure of the protein kinase family [4]. However, severe deviations from the kinase active site consensus sequence and the lack of an ATP-binding domain make it unlikely that mouse NIPK and its counterparts are functional protein kinases. NIPK has been reported to block the activation of serine-threonine kinase Akt/PKB by binding directly to the kinase and participate in the regulation of insulin signaling [5,6]. We and other researchers have shown that NIPK interacts with activating transcription factor 4 (ATF4) and regulates its activity [1,3]. Recently, NIPK has been reported to bind to mitogen-activated protein kinase (MAPK) kinases and control MAPK cascades [7].

Transcriptional upregulation of the *NIPK* gene in response to various stresses like disrupted calcium

☆ *Abbreviations:* ASNS, asparagine synthetase; ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP-homologous protein; EMSA, electrophoretic mobility shift assay; ER, endoplasmic reticulum; NIPK, neuronal cell death-inducible putative kinase; MAPK, mitogen-activated protein kinase; NSRE, nutrient-sensing response element; nt, nucleotide(s).

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metabolism, trophic factor deprivation, glutathione depletion, exposure to dithiothreitol, and hypoxia has been described by several groups [1–3,8], but the induction mechanism and stress-responsive regulatory elements of the gene have not been characterized yet. In the present paper, we have studied the activation of human (h) *NIPK* gene during ER and arsenite stresses, and cloned several *hNIPK* mRNA isoforms synthesized in the cells exposed to thapsigargin. We demonstrate that *hNIPK* promoter segment, consisting of repeated sequences that contain a regulatory element similar to C/EBP-ATF composite site of the *Chop* promoter, mediates upregulation of the gene during the stress response. We also provide evidence that ATF4 can bind to this element and activate the *hNIPK* promoter. The coexpression of NIPK inhibits ATF4 transcriptional activation activity, and thus NIPK may function as a negative feedback regulator of ATF4.

Materials and methods

Mammalian cell culture, transfection, and treatment. Cos-7 and HepG2 cells (obtained from the American Type Culture Collection) were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. Transfection of the cells was carried out by polyethylenimine (ExGen 500 in vitro transfection reagent, Fermentas, Lithuania) or by electroporation, using Gene Pulser II (Bio-Rad, Hercules, CA) at a setting of 950 μF and 180 V (for cos-7) or 200 V (for HepG2). Cells were exposed to 1 μM thapsigargin (Sigma, St. Louis, MO) or to 30 μM sodium arsenite (Sigma) for the time indicated.

cDNA cloning and identification of transcription initiation sites of hNIPK. Total RNA was isolated using the RNeasy kit (Qiagen, Hilten, Germany) from HepG2 cells exposed to thapsigargin for 15 h. The cap structure of the mRNA was replaced with an oligo-ribonucleotide by the oligo-capping method [9,10] and cDNA was synthesized using the GeneRacer kit (Invitrogen, Carlsbad, CA) as follows. Four micrograms of RNA was dephosphorylated with calf intestinal phosphatase (10 U) at 50 °C for 1 h, extracted with phenol:chloroform, and precipitated with ethanol. Next, RNA was treated with tobacco acid pyrophosphatase (0.5 U) at 37 °C for 1 h, extracted with phenol:chloroform, and precipitated again with ethanol. After that, oligoribonucleotide 5′-CGACUGGAGCACGAGGACACUGACAUGG ACUGAAGGAGUAGAAA-3′ was ligated to decapped mRNA with T4 RNA ligase (5 U) at 37 °C for 1 h, and reverse transcription was carried out with SuperScript II Reverse Transcriptase (200 U) at 42 °C for 1 h, using GeneRacer oligo(dT) and random hexamer primers. To clone the full-length *hNIPK* cDNA, 5′ and 3′ fragments of the cDNA were amplified by the gene-specific primer GSP1 (5′-GAGAAC AGGGCTTGGCACCTG-3′) and GeneRacer 5′ primer (5′-CGA CTGGAGCACGAGGACACTGA-3′), and by the gene-specific primer GSP2 (5′-CGAAGCCGCCACCGTATCC-3′) and GeneRacer 3′ primer (5′-GCTGTCAACGATACGCTACGTAACG-3′), respectively. The cDNA fragments were inserted into vector pCR4Blunt-TOPO (Invitrogen) and analyzed by automated DNA sequencing. To identify transcription initiation sites of *hNIPK*, PCR with the *hNIPK*-specific primer GSP3 (5′-CTCCAACCGCTTCTTCCTGGACAG-3′) and GeneRacer 5′ primer was carried out. The products were inserted into pCR4Blunt-TOPO and sequenced.

Northern blot analysis. Total RNA was isolated from HepG2 cells using the RNeasy kit, separated on 1.2% agarose gel containing formaldehyde, and transferred to a positively charged nylon membrane Hybond-N^+ (Amersham, UK). The filters were probed with [^{32}P]dCTP-labeled *hNIPK*, *GAPDH* or *β-actin* cDNA fragments according to standard protocol [11]. I.M.A.G.E. EST clone No. 2900717 used as a *hNIPK* cDNA probe was purchased from Research Genetics (Huntsville, AL). The probes specific to different regions of *hNIPK* exon 1A (named 1A-5′, 1A-repeats, and 1A-3′, which extend from *hNIPK* position −7358 to −7131, −7222 to −7000, and −7020 to −6835, respectively) and to *hNIPK* exon 1B (named 1B, which extends from *hNIPK* position −6718 to −6499) were synthesized by PCR. The hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized to values of *GAPDH*.

Expression plasmids and reporter gene constructs. Expression construct for ATF4 (ATF4-pCG), containing the full-length coding region of ATF4 cDNA in vector pCGN, has been previously described [1]. In order to make an expression plasmid for a dominant negative mutant of ATF4, we introduced 6 amino acid substitutions into the DNA-binding domain of ATF4, as earlier described by He et al. [12], replacing a fragment of ATF4 cDNA encoding amino acid residues ^{292}RYRQKKR^{298} in plasmid ATF4-pCG by a fragment encoding ^{292}GYLEAAA^{298}. Expression construct E2-hNIPK-pCG encoding hNIPK with the N-terminal E2 epitope tag was made by insertion of the full-length coding region of *hNIPK* cDNA into plasmid pCG-3F12 [13].

To analyze promoter activity of *hNIPK* 5′ terminal region, corresponding DNA fragments were amplified by PCR from human genomic DNA and inserted into promoterless firefly luciferase reporter plasmid pGL3-Basic between the *Kpn*I and *Bgl*II sites (constructs −7857/−6940 and −7032/−6940), between the *Kpn*I and *Xho*I sites (constructs −7857/−7033 and −7131/−7033) or between the *Kpn*I and *Mlu*I sites (construct −7857/−7132). The 33-bp repeat of *hNIPK* 5′ terminal region was inserted into plasmid pGL3-Basic between the *Kpn*I and *Xho*I sites (construct wt 33-bp), the 14-bp C/EBP-ATF composite site was inserted into plasmid pGL3-Basic between the *Kpn*I and *Mlu*I sites (construct C/EBP-ATF), and mutant 33-bp repeats (which are identical to the mutant 33-bp repeats used in EMSA) were inserted into plasmid pGL3-Basic into *Ecl*136II site (constructs mut-C/EBP, mut-ATF, mut-5′, and mut-3′). The structure of the constructs was verified by sequencing.

Electrophoretic mobility shift assay. Cos-7 cells were transfected by electroporation with 3 μg of either ATF4-pCG, E2-hNIPK-pCG, empty vector, or their pairwise combinations, and cultured for 24 h before the preparation of cell extracts. The cell extract preparation and electrophoretic mobility shift assay (EMSA) procedures have been described earlier [1]. A 33-nt oligonucleotide identical to the 33-bp repeated sequence of the hNIPK promoter and mutant 33-nt oligonucleotides with base substitutions in the C/EBP half-site (GATTAGC TCCGTCTAGAATCACCCGGACCGGGG) (mutated nucleotides are underlined), in the ATF half-site (GATTAGCTCCGGTTTG CTCTAGACGGACCGGGG), in the 5′ region (GATCTAGA CCGGTTTGCATCACCCGGACCGGGG) or in the 3′ region (GAT TAGCTCCGGTTTGCATCACCCGGTCTAGAG) were labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase, annealed with the complementary strand, and used as the DNA probe. In competition experiments, a 50-fold excess of unlabeled double-stranded oligonucleotide 5′-ACAAAGTACCGTTGCCGGTCGAA-3′, containing the binding site of E2 protein of bovine papillomavirus type 1 [14], was included in the reaction as a nonspecific competitor. For the supershift assay, 2 μg of anti-ATF4 antibody, anti-p300 antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA), or anti-E2 antibody (Quattromed, Estonia) was used.

Luciferase reporter assay. HepG2 cells were cotransfected with 2 μg pGL3-Basic-derived reporter gene constructs containing *hNIPK* 5′ terminal fragments and 0.2 μg pRL-TK (plasmid encoding *Renilla* luciferase). One microgram of expression plasmid for ATF4, DN-ATF4 or hNIPK was included in the transfections when appropriate. Total amounts of transfected DNA were kept constant by adding

corresponding empty vectors. Twenty-two hours after transfection, the cells were exposed to thapsigargin for 18 h, to arsenite for 8 h, or left untreated. After that, luciferase assays were conducted, measuring the activities of both firefly and *Renilla* luciferases with a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Results and discussion

Cloning of hNIPK mRNA isoforms

Hybridization of a blot containing RNA samples isolated from human hepatoblastoma cell line HepG2 to the *hNIPK* cDNA probe (the *hNIPK* coding region originating from I.M.A.G.E. EST clone No. 2900717) revealed that *hNIPK* is expressed in the hepatoblastoma cells cultured under normal conditions, and, similarly to *NIPK* in mouse neuronal cell line GT1-7 [1], the *hNIPK* mRNA level is upregulated in the human hepatoblastoma cells in response to the disruption of calcium homeostasis by thapsigargin (Fig. 1). *hNIPK* expression is also induced in the cells treated with sodium arsenite (Fig. 1). In order to obtain full-length *hNIPK* cDNA and map transcription start sites of *hNIPK* in the stressful conditions, we synthesized a cDNA pool of RNA isolated from thapsigargin-treated HepG2 cells, using the RNA ligase mediated oligo-capping method [9,10]. The method results in the ligation of an oligonucleotide to the 5′ ends of full-length mRNA as a PCR primer docking site, ensuring subsequent amplification of only those cDNA molecules that contain intact 5′ ends of transcripts. 5′ and 3′ pieces of the *hNIPK* cDNA were amplified from the HepG2 cDNA pool and subcloned. The nucleotide sequencing of the clones showed that they represented several isoforms of *hNIPK* mRNA, differing in their 5′ terminal regions (EMBL Nucleotide Sequence Database Accession Nos. AJ697936–AJ697940). The alignment of the nucleotide sequence of the *hNIPK* cDNA clones and the working draft sequence of the human genome revealed that the *hNIPK* gene is organized into four exons interrupted by three introns, and the

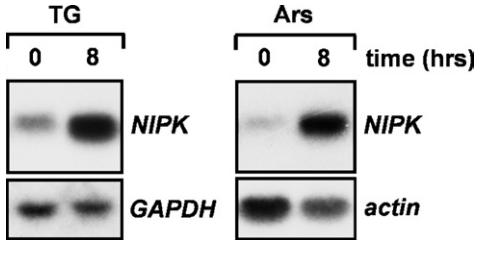

Fig. 1. Thapsigargin and arsenite treatments increase *hNIPK* mRNA level in HepG2 cells. The Northern blot analysis of RNA isolated from the cells that had been treated with 1 μM thapsigargin (TG) or 30 μM sodium arsenite (Ars) for 8 h or left untreated. The blots were hybridized sequentially with the probe covering *hNIPK* coding region (upper panels) and *GAPDH* or *actin* probe (lower panels).

mRNA isoforms contain alternative variants of the first exon (termed 1A and 1B), which are generated by using different transcription initiation regions and splice donor sites (Figs. 2A and B). While exon 1A clones arose from a single splice donor site, exon 1B clones were generated by using four alternative splice donor sites designated as 1B1, 1B2, 1B3, and 1B4 on the basis of their 5′–3′ order (of these four sites, 1B4 appeared to be the major one, used in about 90% of the exon 1B clones). All the exon 1 variants are joined to exon 2 through the same splice acceptor site, located immediately upstream of the translation initiator codon of the hNIPK ORF.

Mapping of hNIPK transcription initiation sites

To study the distribution of transcription start sites, 5′ terminal fragments of *hNIPK* cDNA were amplified from the thapsigargin-treated HepG2 cDNA pool, using *hNIPK*-specific antisense primer GSP3 and GeneRacer 5′primer. The PCR products were subcloned and the nucleotide sequencing of 53 clones (30 clones representing isoform 1A and 23 clones representing isoform 1B) was carried out. As shown in Fig. 2B, the isoform 1A clones initiated at 10 sites in a region extending from position −7257 to −6907 (nucleotides are numbered relative to A of hNIPK translation initiator codon designated as +1) and the isoform 1B clones initiated at nine sites in a region extending from position −6716 to −6663. The G nucleotide at position −6943 and the A nucleotides at positions −6942 and −6667 were identified as cap sites in 9, 8, and 10 clones, respectively, and the rest of the start sites were represented by 1–4 clones. A 33-nucleotide repeated sequence was found at positions −7098 to −7066 and −7065 to −7033 in a fraction of *hNIPK* cDNA isoform 1A clones (Fig. 2B). The inspection of GenBank databases revealed the existence of *hNIPK* sequences containing from two to five copies of the 33-bp repeat, indicating that the *hNIPK* gene is polymorphic relative to the copy number of the repeat.

hNIPK mRNA isoforms are induced differentially in the stressed cells

The Northern blot hybridization of HepG2 RNA to probes specific to various regions of *hNIPK* exon 1A and to exon 1B was carried out to study the level of *hNIPK* mRNA isoforms in normal conditions and during ER and arsenite stresses (Fig. 2C). *hNIPK* mRNA isoform 1A is clearly detectable in unstressed cells and its level is increased about 2-fold in the cells exposed to thapsigargin and about 4-fold in the cells exposed to arsenite (as revealed by hybridization of the blot to probe containing exon 1A region located downstream of the 33-bp repeats). Differently from that, *hNIPK* mRNA isoform 1B signal is very faint in unstressed

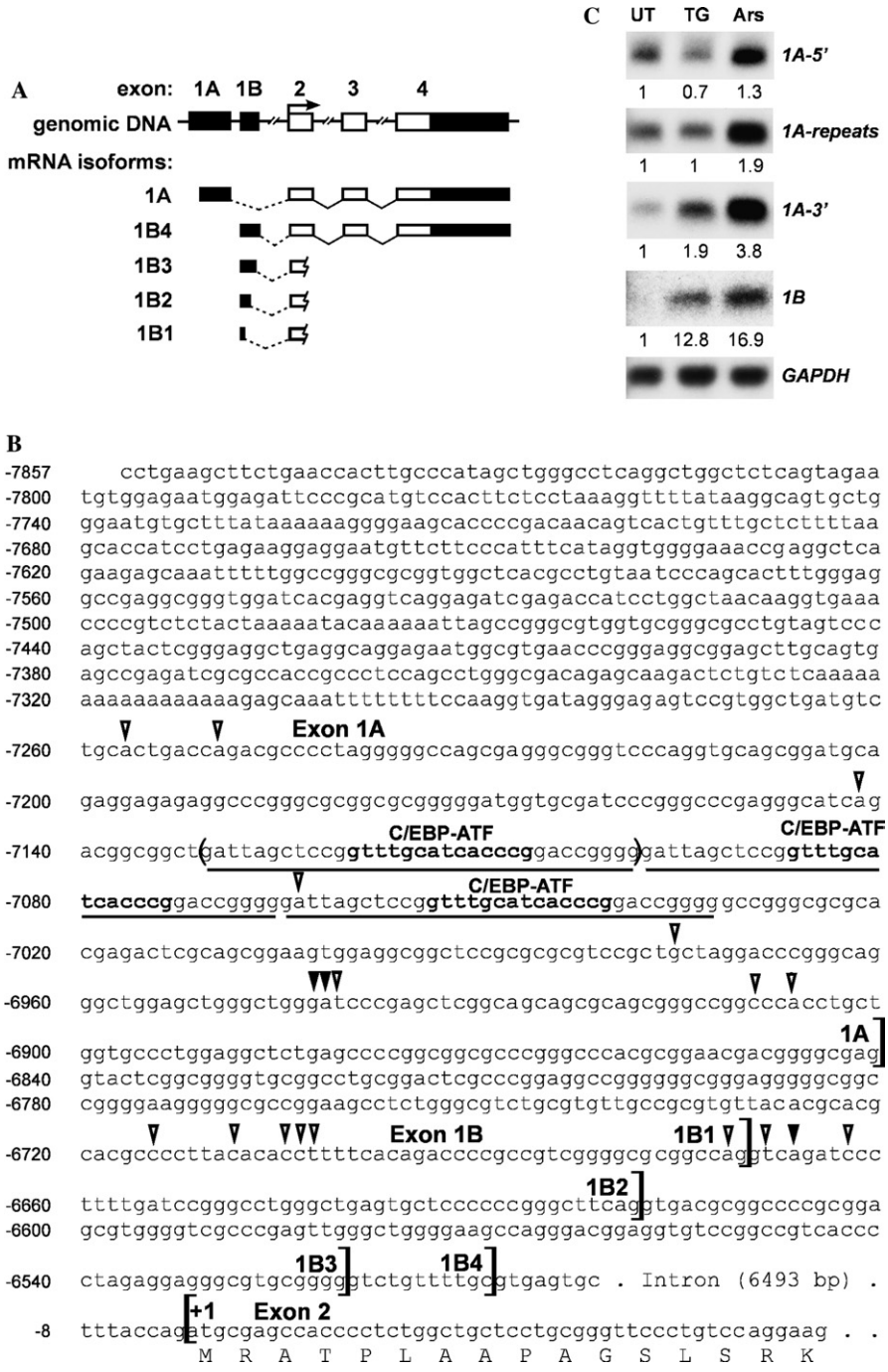

Fig. 2. Characterization of *hNIPK* mRNA isoforms and the 5′ terminal region of the gene. (A) The schematic representation of the *hNIPK* gene exon–intron organization and the structures of the *hNIPK* mRNA isoforms. Exons are represented as boxes (open boxes mark exons encoding hNIPK) and introns are indicated by solid lines. The alternative processing of the first exon is indicated by dotted lines and an arrow marks the translational start site of hNIPK in exon 2. (B) The nucleotide sequence of the 5′ terminal region of the *hNIPK* gene and the mapped transcription initiation sites of the gene in HepG2 cells treated with thapsigargin. The splice donor sites of exons 1A and 1B are indicated by brackets open to the left and the splice acceptor site of exon 2 is indicated by a bracket open to the right. Transcription initiation sites determined by oligo-capping method are marked by vertical arrowheads (filled arrowheads mark abundantly used transcription initiation sites (found in eight or more cDNA clones)). Three copies of the 33-bp repeats (underlined) as found in the human genomic DNA fragment are shown in the figure. One of the repeats, which is missing in the HepG2 cDNA clones (due to allelic variation), is given in parentheses. The C/EBP-ATF composite site sequences are in bold. The deduced amino acid sequence of the N-terminal part of hNIPK is in uppercase letters below the corresponding codons. (C) Northern blot analysis of the induction of *hNIPK* mRNA isoforms in response to thapsigargin and arsenite stresses. The blot containing total RNA isolated from HepG2 cells that had been treated with thapsigargin for 15 h (TG), with arsenite for 10 h (Ars) or left untreated (UT) was hybridized to three *hNIPK* exon 1A probes named 1A-5′, 1A-repeats, and 1A-3′ (covering region upstream of the 33-bp repeats, the 33-bp repeats and short flanking sequences, and region downstream of the 33-bp repeats, respectively), and to exon 1B probe named 1B. Hybridization to *GAPDH* was used as a loading control. The mRNA signals were quantified by a PhosphorImager and amounts of *hNIPK* mRNA isoforms in the stressed cells relative to those in untreated cells (set as 1) were calculated (shown below each band).

cells, but demonstrates strong induction (more than 10-fold) in the stressful conditions. Hybridization of the blot to the probes containing the 5′ terminal part (the region upstream of the 33-bp repeats) and the middle part of exon 1A (the 33-bp repeats and short sequences flanking the repeats) reveals that thapsigargin and arsenite treatments generate no changes or only weak changes in the level of *hNIPK* mRNA isoform 1A molecules extending upstream of the 33-bp repeats.

Identification of hNIPK promoter region regulating activation of transcription in response to thapsigargin and arsenite

In order to characterize regulatory elements of the *hNIPK* promoter, we isolated a 918-bp long human genomic DNA fragment extending from position −7857 to −6940 of *hNIPK* (Fig. 2B) and inserted it into vector pGL3-Basic, encoding the modified firefly luciferase. The resulting construct was transiently transfected into HepG2 cells, and luciferase activity was measured in the cells treated with thapsigargin or arsenite or left untreated. Compared to the promoterless pGL3-Basic, the construct −7857/−6940 produced more than a 200-fold increase in the luciferase activity in untreated cells, and about 500- and 900-fold increase in the luciferase activity in the cells exposed to thapsigargin and arsenite, respectively (Fig. 3A). Thus, the fragment from −7857 to −6940 contains promoter elements involved in basal transcription as well as in induction of transcription in response to thapsigargin and arsenite.

The nucleotide sequence analysis of the *hNIPK* fragment −7857/−6940 showed that there are three tandemly arranged 33-bp-long repeats at positions −7131 to −7033 (Fig. 2B). A search of the nucleotide sequence of the fragment −7857/−6940 for motifs similar to previously identified stress-related promoter elements revealed that the middle part of the 33-bp repeat exhibits similarity to a regulatory element in the *Chop* promoter, termed C/EBP-ATF composite site (named also amino acid response element) [15–17], and to a regulatory element in the *Asns* promoter, termed nutrient-sensing response element (NSRE)-1 [18,19] (Fig. 3B), which are both involved in activation of the genes in response to various stresses, including ER and arsenite stresses [15,19–22]. To examine whether the region containing tandem 33-bp repeats regulates *hNIPK* activation during ER and arsenite stresses, luciferase reporter constructs with the deletion mutants of the *hNIPK* promoter region were created and analyzed in HepG2 cells (Fig. 3A). The fragment extending from position −7857 to −7033, which covers the 33-bp repeats and the region upstream of it, promotes about 60-, 150-, and 300-fold increase in luciferase activity relative to the promoterless control in untreated, thapsigargin-treated, and arsenite-treated cells, respectively, and thus has retained responsiveness to thapsigargin (2.6-fold induction) and arsenite (5.6-fold induction). Similarly, the construct −7131/−7033, which contains the 33-bp repeats only, exhibits the induction of luciferase expression by thapsigargin and arsenite (2.7- and 9.7-fold, respectively). In contrast, constructs containing fragments upstream (from position −7857 to −7132) and downstream (from position −7032 to −6940) of the 33-bp repeats have lost the ability to activate transcription in response to thapsigargin and arsenite, and these constructs also reveal lower basal transcriptional activity than the constructs containing the 33-bp repeats. Thus, the segment consisting of tandemly arranged 33-bp repeats is necessary for the thapsigargin and arsenite responsiveness of the *hNIPK* promoter.

In order to study whether the C/EBP-ATF composite site is important for the stimulation of transcriptional activity by thapsigargin and arsenite, and to assess the contribution of regions flanking the site within the 33-bp repeat, luciferase reporter constructs driven by the single 33-bp repeat and its mutants were analyzed in the stressed HepG2 cells (Fig. 3C). Both thapsigargin and arsenite induce luciferase expression in the case of wild-type 33-bp repeat, and responsiveness to these agents is lost by the mutation of ATF as well as C/EBP part of the C/EBP-ATF composite site and by the mutation of nucleotides preceding the C/EBP-ATF composite site. Differently from that, the mutation of nucleotides flanking the C/EBP-ATF composite site in the 3′ direction increases the inducibility of reporter gene expression by thapsigargin and arsenite, suggesting that a binding site of a transcriptional repressor may reside in this region (alternatively, the increased responsiveness may be caused by enhanced binding of positive transcriptional regulator(s) to the mutant promoter segment). Thus, the C/EBP-ATF composite site as well as regions flanking the site within the 33-bp repeat are involved in the control of *hNIPK* promoter in the stressful conditions.

ATF4 binds to the 33-bp repeat and activates hNIPK promoter

It has previously been demonstrated that the upregulation of *Chop* and *Asns* transcription in the stressful conditions is mediated by ATF4, which interacts with the C/EBP-ATF composite site in the *Chop* promoter and with NSRE-1 in the *Asns* promoter [15,23]. ATF4 is synthesized at a barely detectable level in HepG2 cells cultured under normal conditions, but is greatly increased in the cells exposed to thapsigargin or arsenite (Fig. 4A). In order to study whether ATF4 can bind to the 33-bp repeated sequence of the *hNIPK* promoter, cos-7 cells were transfected transiently with an expression construct for ATF4 or empty vector and cell extracts were analyzed by EMSA. Compared to the

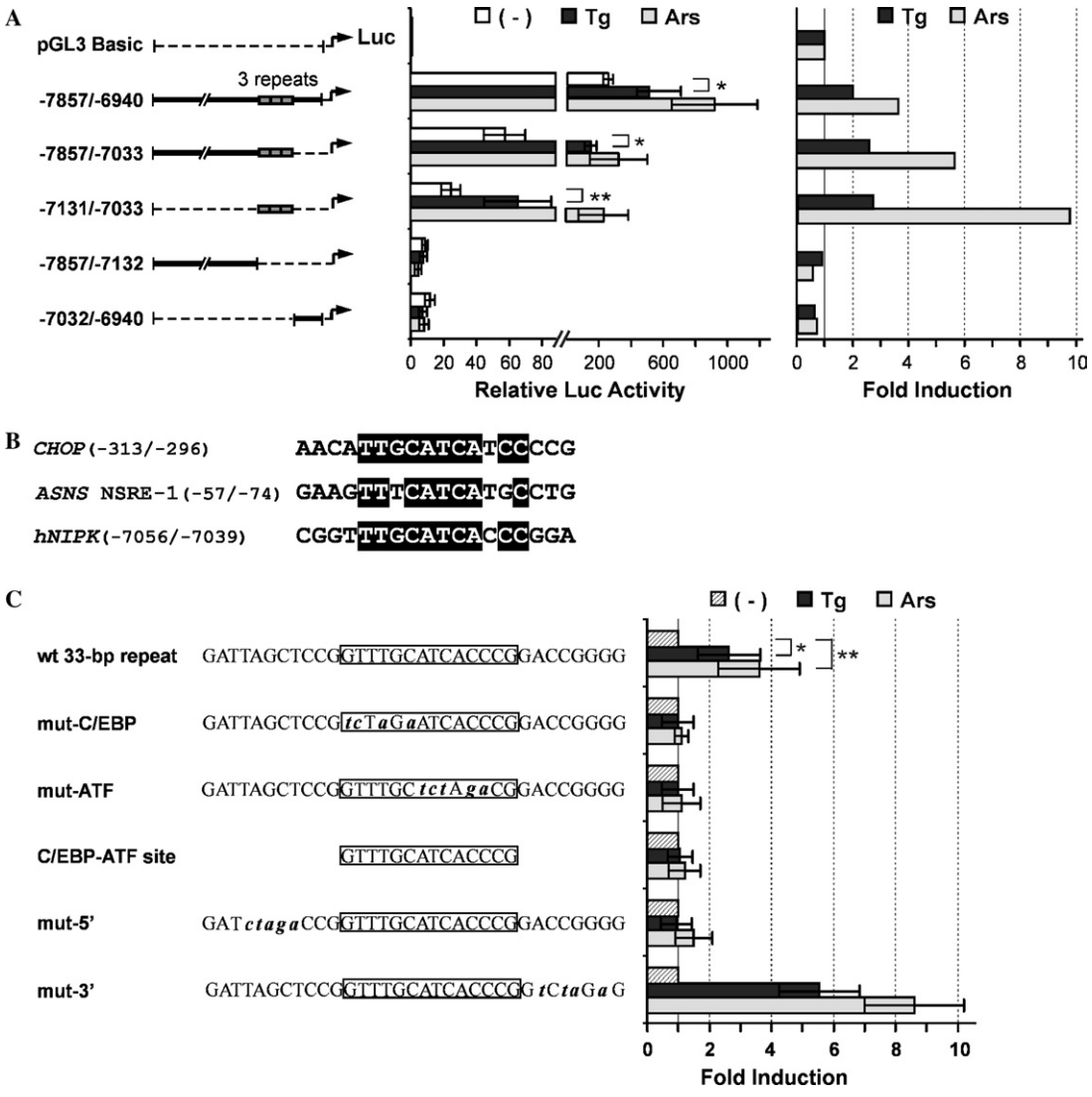

Fig. 3. Identification of *hNIPK* promoter region regulating activation of transcription in response to thapsigargin and arsenite. (A) The deletion analysis of *hNIPK* promoter activity in HepG2 cells. The left panel is a schematic representation of a 918 bp *hNIPK* promoter region and its deletion mutants, analyzed by luciferase reporter assay. The deleted regions are marked by dashed lines and the 33-bp repeats are indicated by shaded boxes. The middle panel shows relative luciferase activity in untreated (open bars), thapsigargin-treated (solid bars), and arsenite-treated cells (gray bars). The level of luciferase activity in cells transfected with promoterless reporter plasmid (pGL3-Basic) was set as 1. The means ± SD values were calculated from the results of at least four independent experiments (*$P < 0.001$ and **$P < 0.005$ by two-tailed t test). The right panel shows the fold induction, defined as the ratio of the relative luciferase activity of thapsigargin- or arsenite-treated cells to untreated cells. (B) The comparison of the nucleotide sequence of the *Chop* C/EBP-ATF composite site, *Asns* NSRE-1, and the middle part of the 33-bp repeat of the *hNIPK* promoter. Nucleotides shared by the *hNIPK* and *Chop* or *Asns* sequences are shown on solid background. (C) Responsiveness of the wild-type and mutant 33-bp repeats to thapsigargin and arsenite stresses. HepG2 cells were transfected with luciferase reporter constructs driven by the wild-type (wt) 33-bp repeat, mutant 33-bp repeats (mut-C/EBP, mut-ATF, mut-5′, and mut-3′) or 14-bp C/EBP-ATF composite site. The C/EBP-ATF composite site within the nucleotide sequence of the 33-bp repeat is boxed and mutated nucleotides are in lower-case letters. The cells were treated with thapsigargin for 18 h, with arsenite for 8 h or left untreated. The fold induction (defined as the ratio of the relative luciferase activity of thapsigargin-treated (solid bars) or arsenite-treated cells (gray bars) to untreated cells (striped bars)) was calculated from the results of at least four independent experiments (*$P < 0.001$ and **$P < 0.01$ by two-tailed t test).

control experiment with the empty vector, the incubation of ^{32}P-labeled 33-bp repeat with the extract of cells transfected with expression construct for ATF4 produces two markedly increased complexes, which are competed by excess unlabeled 33-bp repeat, but not by an unrelated oligonucleotide (Fig. 4B, lanes 1–4). The antibody supershift analysis reveals that both these complexes contain ATF4 (Fig. 4B, lane 5). The mutation of ATF as well as C/EBP portion of the C/EBP-ATF composite site abrogates the complex formation between ATF4 and the DNA probes (formation of an unrelated protein–DNA complex is observed, which does not contain ATF4 as revealed by the antibody supershift assay). At the same time, the mutation of nucleotides flanking

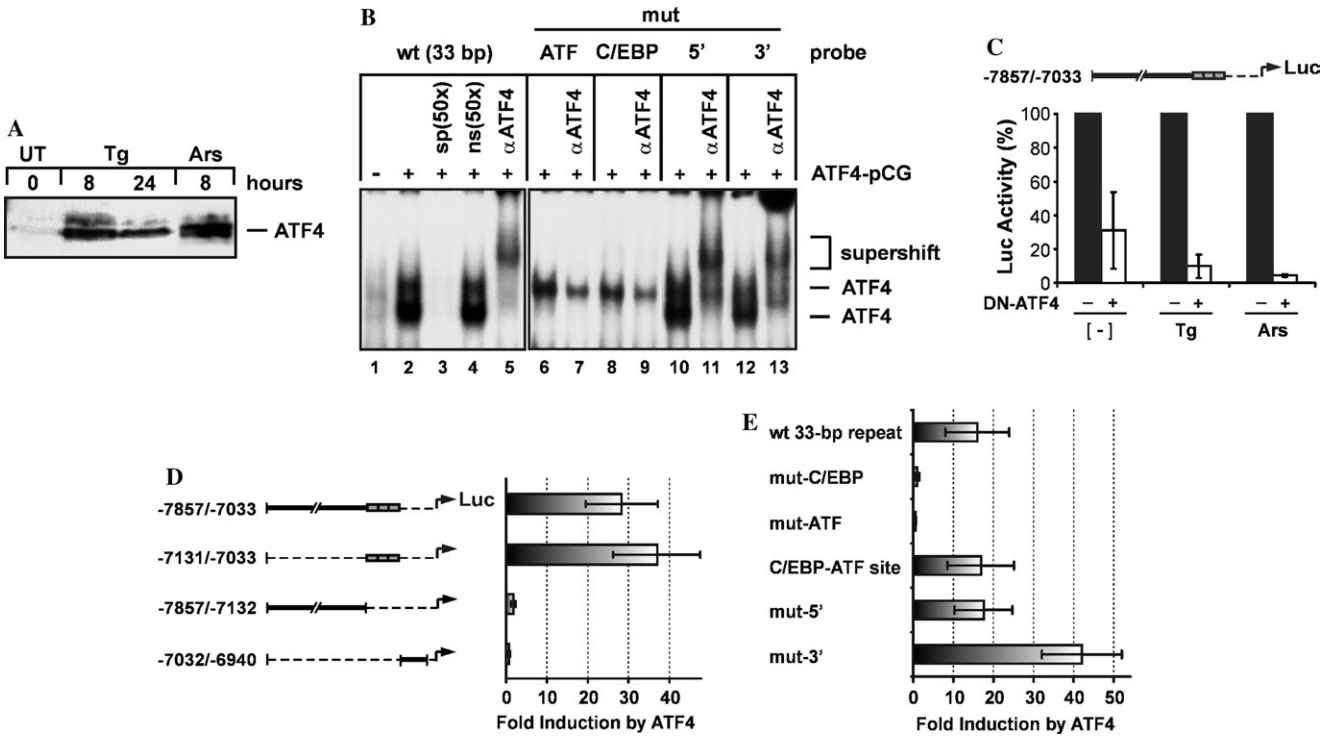

Fig. 4. ATF4 binds to the 33-bp repeat and activates *hNIPK* promoter. (A) ATF4 level is increased in HepG2 cells exposed to thapsigargin or arsenite. The immunoblot analysis of ATF4 protein content of the cells that had been treated with thapsigargin (Tg) or arsenite (Ars) for the indicated period of time or left untreated (UT). Twenty micrograms of total cellular protein was loaded on each lane and the blot was probed with polyclonal anti-ATF4 antibody. (B) The EMSA of the radiolabeled wild-type 33-bp repeat (lanes 1–5) and mutant 33-bp repeats (lanes 6–13) with extracts made of cos-7 cells transfected with the expression construct for ATF4 (lanes 2–13) or empty vector (lane 1). Mutant 33-bp repeats contain nucleotide substitutions in the ATF (lanes 6 and 7) or in the C/EBP half-site of the C/EBP-ATF composite site (lanes 8 and 9) or in region flanking the site in 5′ (lanes 10 and 11) or 3′ direction (lanes 12 and 13). A 50-fold excess of unlabeled 33-bp repeat oligonucleotide (lane 3) or unrelated oligonucleotide (containing the binding site of E2 protein of BPV1) (lane 4) was added to the extracts as a specificity control. Anti-ATF4 antibody (lanes 5, 7, 9, 11, and 13) was used for the supershift analysis and the supershifted complexes are indicated. (C) A dominant-negative mutant of ATF4 (DN-ATF4) inhibits the activity of *hNIPK* promoter. HepG2 cells were cotransfected with luciferase reporter plasmid driven by the *hNIPK* promoter region −7857/−7033 along with the expression construct for DN-ATF4 (open bars) or empty vector (solid bars), and treated with thapsigargin for 18 h (marked by Tg below the bars), with arsenite for 8 h (marked by Ars) or left untreated (marked by [-]). The percent luciferase activity was calculated by arbitrarily defining reporter activity in the absence of DN-ATF4 as 100%. The results are averages with SD of three experiments. (D) ATF4 activates the *hNIPK* promoter through the 33-bp repeats. HepG2 cells were cotransfected with luciferase reporter constructs driven by *hNIPK* promoter or its deletion mutants along with the expression construct for ATF4 or empty vector. The left panel is a schematic representation of the *hNIPK* promoter region and its deletion mutants, analyzed by luciferase reporter assay. The deleted regions are marked by dashed lines and the 33-bp repeats are indicated by shaded boxes. The right panel shows the fold induction of luciferase activity of the cells transfected with the expression plasmid for ATF4, relative to the cells transfected with the empty vector. The means ± SD values were calculated from the results of at least four independent experiments. (E) The mutation of the C/EBP-ATF composite site within the 33-bp repeat abrogates stimulation of transcription by ATF4. HepG2 cells were cotransfected with luciferase reporter constructs driven by the wild-type (wt) 33-bp repeat, mutant 33-bp repeats (mut-C/EBP, mut-ATF, mut-5′, and mut-3′) or 14-bp C/EBP-ATF composite site along with the expression construct for ATF4 or empty vector. The fold induction of luciferase activity of the cells transfected with the expression plasmid for ATF4 (relative to the cells transfected with the empty vector) was calculated from the results of at least four independent experiments.

the C/EBP-ATF composite site within the 33-bp repeat retains ATF4 binding to the DNA probes (Fig. 4B, lanes 10–13).

The ability of ATF4 to form the complex with the 33-bp repeat of the *hNIPK* promoter suggests that ATF4 may participate in the *hNIPK* gene regulation. To investigate this potential function, we examined whether the expression of ATF4 or a dominant negative mutant of ATF4 (DN-ATF4) [12,23] affects the expression level of the luciferase reporter gene driven by the *hNIPK* promoter. The transfection of HepG2 cells with an expression construct for DN-ATF4, along with the *hNIPK* promoter construct −7857/−7033, diminished luciferase activity by 70% in untreated cells (relative to the control without DN-ATF4), and by 90% and 95% in the cells exposed to thapsigargin and arsenite, respectively (Fig. 4C). Opposite to the inhibitory effect of DN-ATF4, the transfection of HepG2 cells with an expression plasmid for ATF4, along with the *hNIPK* promoter construct −7857/−7033, strongly stimulates the reporter gene expression (more than 20-fold) (Fig. 4D). Even greater enhancement (more than 30-fold) is observed

in the case of the *hNIPK* fragment −7131/−7033 containing only the 33-bp repeats. At the same time, fragments of the *hNIPK* promoter region upstream (−7857/−7132) and downstream (−7032/−6940) of the 33-bp repeats reveal no response to ATF4. Thus, ATF4 activates the *hNIPK* promoter through the region consisting of the 33-bp repeats. To confirm that the C/EBP-ATF composite site within the 33-bp repeat is essential for the stimulation of reporter activity by overexpressed ATF4, luciferase constructs driven by the single 33-bp repeat and its mutants were analyzed (Fig. 4E). Consistent with the results of EMSA, the base substitutions in the C/EBP-ATF composite site abolish the stimulation of reporter activity by ATF4, but the mutation of nucleotides preceding the C/EBP-ATF composite site has no effect. The mutation of nucleotides flanking the C/EBP-ATF composite site in the 3′ direction increases the stimulatory effect of ATF4, similarly to the increase of responsiveness to the stress-inducing agents.

Collectively, these results indicate that ATF4 is able to interact with the 33-bp repeated sequence through the C/EBP-ATF composite site and participate in the regulation of *hNIPK* promoter.

hNIPK inhibits basal activity, and the stress- and ATF4-induced activity of the hNIPK promoter

We have previously reported that mouse NIPK interacts with ATF4 and inhibits its ability to activate transcription through the CRE/ATF site [1]. Also, hNIPK binds to ATF4 in the yeast two-hybrid system and the formation of hNIPK–ATF4 complex in mammalian cells can be detected by coimmunoprecipitation ([3] and our data not shown). In order to explore whether hNIPK affects reporter gene expression driven by *hNIPK* promoter, HepG2 cells were cotransfected with the *hNIPK* promoter construct −7857/−7033 and an expression plasmid for hNIPK or empty vector, and luciferase activity was measured in the cells exposed to thapsigargin or arsenite, left untreated, or cotransfected additionally with the expression plasmid for ATF4. Similarly to DN-ATF4, hNIPK significantly reduces luciferase expression in unstressed cells (about 40%) and in thapsigargin- or arsenite-stressed cells (about 75% and 70%, respectively) (Fig. 5A). The transactivation of *hNIPK* promoter by overexpressed ATF4 is almost totally (99%) blocked by coexpressed hNIPK (Fig. 5A). These results suggest that NIPK may function as a negative feedback regulator of ATF4.

In order to study whether hNIPK affects ATF4 ability to bind to the 33-bp repeated sequence of the *hNIPK* promoter, cos-7 cells were transfected transiently with expression constructs for hNIPK and ATF4, and cell extracts were analyzed by EMSA (Fig. 5B). Compared to the cells transfected with expression construct for ATF4 alone (lanes 4 and 5), the cells coexpressing hNIPK and ATF4 reveal no difference in the pattern of the retarded bands that are supershifted by anti-ATF4 antibody (lanes 6 and 7). The supershift experiment with anti-E2 antibody (recognizing the epitope tag linked to hNIPK) produces a faint slower-migrating band in the cells coexpressing hNIPK and ATF4 (lane 8) that is not observed in the control experiment with unrelated anti-p300 antibody (lane 9). These results indicate that the coexpression of hNIPK does not inhibit ATF4 binding to the 33-bp repeat and that the majority of ATF4 complexes with the 33-bp repeat do not contain hNIPK (differently from that, mouse NIPK is present in the majority of ATF4-CRE/ATF site oligonucleotide complexes in the EMSA of cells coexpressing ATF4 and mouse NIPK

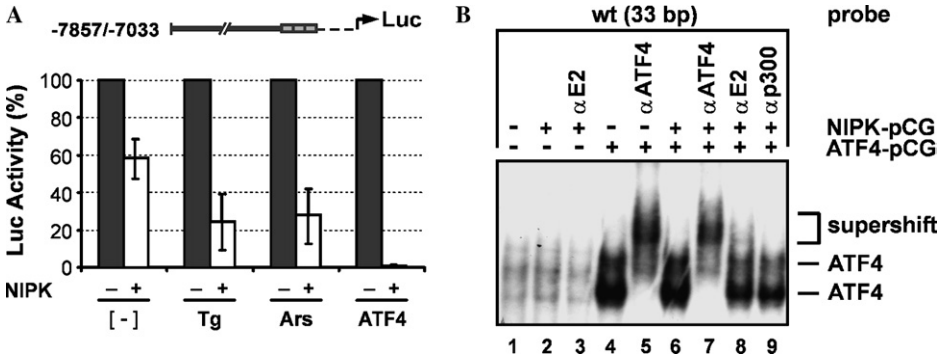

Fig. 5. hNIPK inhibits basal activity, and the stress- and ATF4-induced activity of the *hNIPK* promoter. (A) The results of luciferase assay of HepG2 cells cotransfected with luciferase reporter plasmid driven by the *hNIPK* promoter region −7857/−7033 along with the expression construct for hNIPK (open bars) or the empty vector (solid bars). The cells were treated with thapsigargin for 18 h (marked by Tg below the bars), with arsenite for 10 h (marked by Ars), left untreated (marked by [-]), or transfected additionally with the expression construct for ATF4 (marked by ATF4). The percent of luciferase activity was calculated by arbitrarily defining reporter activity in the absence of hNIPK as 100%. The results are averages with SD of at least three experiments. (B) Coexpression of hNIPK does not inhibit ATF4 binding to the 33-bp repeat of *hNIPK* promoter. EMSA of the radiolabeled 33-bp repeat and extracts made of cos-7 cells transfected with empty vector (pCG) (lane 1), E2-hNIPK-pCG (lanes 2 and 3), ATF4-pCG (lanes 4 and 5), or cotransfected with E2-hNIPK-pCG and ATF4-pCG (lanes 6–9). Anti-E2 antibody (lanes 3 and 8), anti-ATF4 antibody (lanes 5 and 7), and anti-p300 antibody (lane 9) were used for supershift analysis.

[1]). Further studies are needed to uncover the mechanism how the NIPK proteins inhibit ATF4.

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