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Bone Morphogenetic Protein-2 Induces Expression of Murine Zinc Finger Transcription Factor ZNF450

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Abstract The bone morphogenetic protein-2 (BMP-2) is a potent secreted factor that promotes osteoblast differentiation during development. Exposure to BMP-2 is sufficient to cause a lasting change in cell fate presumably by activating specific target genes. To identify genes downstream of BMP-2 we treated the murine pluripotent embryonic cell line, C3H10T1/2 that can be induced to form an osteoblastic phenotype, with 100 ng/ml BMP-2 for 24 h. Using suppression subtractive hybridisation we found the novel zinc finger transcription factor, ZNF450 was upregulated. The single-copy *ZNF450* gene spans 15.6 kb on chromosome 10B1 and consists of seven exons, the first of which is untranslated. The open reading frame encodes a 710 reside protein. Analysis of the protein sequence reveals a highly conserved amino-terminal BTB/POZ dimerisation domain, an AT-hook motif, and eight C2H2 zinc fingers. Library screening identified a second mRNA isoform encoding a short protein isoform with one zinc finger. Using reverse transcriptase-real time PCR to measure mRNA expression we found that ZNF450, Runx2/Cbfa-1, and Sp7/osterix were induced by BMP-2 after 4 h in C2C12 myoblast cells. Treatment of C2C12 cells with BMP-2 causes a shift from a myoblastic to osteoblastic phenotype. ZNF450 was upregulated three to fivefold after 24 h in C3H10T1/2 cells and required 100 ng/ml BMP-2. Expression of the 3 kb major transcript was highest in liver, testis, and kidney. However, ZNF450 mRNA was found also in a wide range of adult tissues. The consistent induction of ZNF450 by BMP-2 after 4 h in three murine pluripotent cell lines suggests that ZNF450 may play a role in the BMP-2 signalling pathway. J. Cell. Biochem. 94: 202–215, 2005.

Key words: ID1; N-CoR; SMRT; histone deacetylase; MAZR/ZNF278; myoneurin

The bone morphogenetic proteins (BMPs) are potent secreted factors that promote osteoblast differentiation during development and bone remodelling. In vivo application of BMPs can induce bone formation and recombinant human BMPs have been approved for accelerating bone fusion in slow-healing fractures (reviewed in [Nakashima and Reddi, 2003]), but the molecular events downstream of BMP-2 signalling that result in tissue-specific gene expression and skeletal development have been only partially elucidated. BMP-2 has been shown to induce several transcription factors that promote osteoblastic differentiation such as Runx2/Cbfa1, Sp7/osterix and Dlx5 [Ducy et al., 1997; Xu et al., 2001; Nakashima et al., 2002; Milona et al., 2003]. In addition, the negative regulator ID1 (inhibitor of DNA binding 1, a dominant negative inhibitor of basic helix-loop-helix DNA binding proteins) has been shown to be directly responsive to BMP stimulation of C3H10T1/2 cells [Hollnagel et al., 1999].

To identify additional factors that may regulate osteoblast differentiation we utilised the pluripotent embryonic cell line C3H10T1/2 as a

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model system [Reznikoff et al., 1973]. On BMP-2 stimulation C3H10T1/2 cells initially undergo differentiation towards a chondrogenic phenotype and subsequently towards an osteogenic phenotype [Katagiri et al., 1990; Wang et al., 1993; Shea et al., 2003]. To achieve this, we used suppression subtraction hybridisation [Diatchenko et al., 1996] to generate a cDNA library enriched in genes upregulated by 100 ng/ml BMP-2 after 24 h. One of the subtracted library clones, named bone morphogenetic protein-induced factor-1, Bif-1, was identified as being part of a novel zinc finger transcription factor, ZNF450. Here, we describe a BTB-zinc finger transcription factor, ZNF450, that may play a role in the BMP-2 signalling pathway.

MATERIALS AND METHODS

Cell Culture

The murine cell lines C3H10T1/2, C2C12, MC3T3-E1, and NIH-3T3 fibroblasts were obtained from the ATCC. The cells were cultured in Dulbecco's modified Eagle's medium supplemented and 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Groningen, The Netherlands) supplemented with 10% new born bovine serum (Sigma, Poole, UK) at 37°C under 5% CO_2 in a humidified incubator. Prior to BMP-2 treatment, cells were seeded at a density of approximately 40,000 cells/cm^2, left for 24 h, transferred to a low serum media containing 0.5% new born bovine serum (Globepharm, Guilford, UK), left for a further 24 h and then treated with fresh media with or without human recombinant BMP-2 (Wyeth corporation, Cambridge, MA).

Generation of a BMP-2 Treated C3H10T1/2 Subtracted cDNA by Subtractive Suppression Hybridisation (SSH)

SSH was performed using the PCR-Select Subtraction protocol (Clontech, Basingstoke, UK) according to the manufacturer's recommendations. Briefly, double stranded cDNA from BMP-2 treated C3H10T1/2 cells were used as the tester and cDNA from untreated C3H10T1/2 cells was used as the driver using a ratio of 1:30. Differentially expressed genes were amplified from the subtracted cDNA using suppression PCR. A GeneAmp 2400 (PE Biosystems, Warrington, UK) was used for thermal cycling. The conditions used were 94°C for 10 s, 68°C for 30 s, and 72°C for 90 s, for 30 cycles (the initial PCR) and 12 cycles (for the nested PCR). PCR amplicons from the tester, driver, and subtracted cDNA were electrophoresed on a 1% agarose gel and prominent amplicons specific to the subtracted cDNA were excised from the gel.

Cloning and DNA Sequencing

The amplicons from the subtracted cDNA were ligated into the pGem-T vector (Promega, Southampton, UK), a TA cloning system, and transformed into JM109 competent cells (Promega). Subtracted clones were plated on to LB media agar plates supplemented with 50 μg/ml ampicillin which were treated with 40 mg/ml 5-bromo-4-chloro-3-indol-β-D-galactopyranoside (Promega) dissolved in dimethylformamide. Agar plates were incubated overnight at 37°C. Positive colonies containing inserts were inoculated into LB media containing ampicillin. Isolated plasmids were sequenced directly using the big dye terminator cycle sequencing ready reaction kit and AmpliTaq DNA polymerase FS and run on an ABI 377 automated DNA sequencers (both from PE Applied Biosystems).

Expression of ZNF450 mRNA by Real Time PCR

Cell line cDNA was analysed for the relative expression of ZNF450, beta-actin mRNA, and 18S RNA by reverse transcriptase-real time PCR. Total RNA was extracted from cells using guanidine thiocyanate and treated with DNase-I to remove any contaminating genomic DNA (Total RNA isolation system, Promega). Total RNA was reverse transcribed with M-MLV RNase H- reverse transcriptase (SuperScript. III, Invitrogen) using random hexamers. PCR was initially carried out on a 7900HT ABI PRISM Sequence Detection System and subsequently on a Rotor Gene 3000 utilising a CAS-1200 robotic precision liquid handling system (Corbett Research, Mortlake, Australia) using both SYBR Green I double-stranded DNA binding dye and 5′-nuclease, Taqman, assays (Applied Biosystems, Warrington, UK). cDNA derived from 50 ng of total RNA from each cell type was amplified by PCR using Taq Gold polymerase using PCR primers to a final concentration of 50 nM and probes to a final concentration of 200 nM. The primers were: mouse ZNF450 long isoform CAGGATCCACCGAGGAGAAA and GGCACTGGAGTCAGAGAAGCA and probe 5′-Fam-CCATACTCCTGCAGCATCTGTGGCA-Tamra-3′ producing a 70 bp amplicon, the location of exon 6/7 boundary is underlined;

ZNF450 short isoform GCCACACGGGTAACTTCGA and CGTCCCAACCACAATTCCA and probe 5′-Fam-CAACCAATCGCACCACGTCTGGAA-Tamra-3′ producing a 70 bp amplicon, the location of the exon 2/intron 2 boundary is underlined. To compare the expression levels of both isoforms of ZNF450 a common forward primer, AAGATGACCAGAGTACTGCCAAGAG; a long protein isoform reverse primer TCTGGAAATCGAAGTTACCCGT and a short protein isoform reverse primer AAGGGTCGTTCCCCCGT were used in a SYBR Green I assay, giving 148 and 153 bp amplicons, respectively. For the SYBR assay beta-actin was used as the control gene as previously described [Edgar and Polak, 2002]; for the 5′-nuclease assay the control was ribosomal RNA reagents with VIC labelled probe (Applied Biosystems) producing a 187 bp amplicon. The primers for other BMP-2 regulated genes were: ID1 GCGGCCGAGGTGAGGT and CTATGCGCCTGAAAAGTAAGGAA producing a 103 bp amplicon; Runx2, AGAAGAGCCAGGCAGGTGCTT and TTCGTGGGTTGGAGAAGCG producing a 102 bp amplicon; Sp7 CACAGCTTCCATCTGGCCTC and TTCGGGAAAACGGCAAATAG producing a 91 bp amplicon. The amplification conditions were: a 10 min hot start to activate the polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The number of cycles required for the fluorescence to become significantly higher than background fluorescence (termed cycle threshold [C_t]) was used as a measure of abundance. A comparative C_t method was used to determine gene expression. Expression levels in each cDNA sample were normalised to the expression levels of the control gene (ΔC_t). The ratios of ZNF450 mRNA/control gene RNA from each cDNA were standardised to that of the untreated cells, which was taken as 100% ($\Delta\Delta C_t$). The formula $E^{-\Delta\Delta Ct}$ was used to calculate relative expression levels where E is the efficiency of the PCR per cycle. For the SYBR assay amplification specificity was confirmed by melting curve analysis and agarose gel electrophoresis.

For the determination of ZNF450 mRNA expression in tissues, sixteen adult mouse tissue cDNAs (BD Clontech) generated from polyA$^+$ selected RNA and reverese transcribed using an oligo-dT primer. Approximately 4.0 ng of cDNA were used for each PCR. Expression levels in each tissue cDNA sample were normalised to the expression levels of the housekeeping gene β-actin (ΔC_t). The ratios of ZNF450/β-actin mRNA from each tissue were standardised to that of ZNF450 in kidney (long isoform) and skeletal muscle (short isoform) which was taken as 100% ($\Delta\Delta C_t$).

Northern-Blot Analysis

For examining ZNF450 expression in C3H10T1/2 cells 20 μg total RNA was subjected to electrophoresis in a formaldehyde-agarose gel transferred to nitrocellulose and hybridised with a [alpha-^{32}P]dCTP-labelled cDNA ZNF450 probe corresponding to exons 1 and 2. A murine G3PDH probe was used as a control gene. A northern blot of mouse poly(A)$^+$ RNA from different tissues with loading normalised to the housekeeping gene beta-actin (BD Biosciences) was hybridised with the ZNF450 probe. Stringency washes were in 0.2× NaCl/Cit at 65°C for 20 min. To investigate the role of protein synthesis in the BMP-2 induction of ZNF450 C3H10T1/2 cells were pretreated with media containing 0.5% new born bovine serum with or without 10 μM cycloheximide for 30 min, followed by the same media with or without 100 ng/ml BMP-2 for 24 h and the cells harvested for total RNA extraction. Autoradiographs of the RNA blots were quantified using a densitometry and the AlphaImager 2000 programme.

Statistics

Statistically significant changes in gene expression were determined using the *t*-test on data from three replicate experiments.

RESULTS

ZNF450 Proteins

The clone named bone morphogenetic protein induced factor-1 (Bif1) was isolated by SSH from C3H10T1/2 cells stimulated with 100 ng/ml BMP-2 for 24 h. The sequence of the murine Bif1 cDNA was identical to the sequence of clone MGC:63306 (GenBank accession no. AAH50933 [Strausberg et al., 2002]) that encodes the ZNF450 protein that has 710 residues with a calculated molecular mass 78,751 Da and an isoelectric point 8.31. The initiation methionine ATG codon corresponds to the Kozak initiation consensus sequence. Database searches identified the human homologue as the protein encoded by clone KIAA0441 isolated from a brain cDNA library [Ishikawa et al., 1997]. Although the rat cDNA has not been cloned the homo-

logous gene is located at 20q12 (GenBank accession no. AC129792) and the predicted cDNA (accession no. BK001278) has an ORF of 705 residues. Similarly, a zebrafish gene is located on clone CH211-232I12 (accession no. BX936450) and the predicted cDNA (accession no. BK005157) has an ORF of 672 residues. Analysis of the four vertebrate proteins reveals a highly conserved amino-terminal BTB or POZ domain (Broad-Complex, tramtrack, and bric a brac or Pox virus, and zinc finger), an AT-hook domain and eight C2H2 zinc fingers (Fig. 1A). A comparison of the murine ZNF450 protein with that of man shows they have 80% identity and 92% similarity. Partial homologous ESTs of ZNF450 were also found in other mammals, *Bos taurus*, *Sus scrofa*, *Canis familiaris*; birds (*Gallus gallus*); amphibians (*Xenopus laevis*), and teleosts (*Takifugu rubripes* and *Oryzias latipes*) suggesting that expression of ZNF450 is restricted to the Euteleostomi (bony vertebrates).

Using the Bif1 cDNA as a probe we also isolated from a mouse cDNA library a clone that encodes a short protein isoform of ZNF450/Bif1 (GenBank accession no. AF263010). This cDNA is generated by exons 1 and 2 and the 5′ end of intron 2 and has a classical polyadenylation signal and is polyadenylated. The short protein isoform encodes the BTB and AT-hook domains and the first zinc finger. The short protein isoform has 354 residues, a calculated molecular mass 40,007 Da and an isoelectric point 6.05. The short protein isoform is also found in humans (on clone MGC:45124, accession no. BC036731). Many ESTs corresponding to the short protein isoform are found in mouse, man, and pig suggesting that the short isoform has a biological role. However, a comparison of the carboxy-terminal regions of the short protein isoforms from different species shows that there is little homology in the sequences encoded by intron 2 (Fig. 1B). Recently, another atypical mouse ZNF450 mRNA isoform has been sequenced from osteoblasts (accession number AAH55367 [Strausberg et al., 2002]) that, on comparison with the ZNF450 gene, differs by utilising a cryptic donor splice site 16 bp into intron 4 creating a frame-shift and has skipped exon 6, encoding a theoretical protein with six zinc fingers.

ZNF450 Gene Structure

By comparing the ZNF450 cDNA sequence with that of the mouse genome its gene structure can be determined. The single-copy murine ZNF450 gene is located on chromosome 10B1 (accession NT_039492). There is a 454 bp CpG island (67% GC rich) that encompasses all of the untranslated-exon 1 (Fig. 2). The whole gene spans 15.6 kb and has seven exons. All splice donor/acceptor sites contained consensus GT/AG dinucleotides. The location of all five exon/exon boundaries are conserved between mice, rat, and humans. Exon 1 is untranslated, exon 2 encodes the BTB and AT-hook domains and the first zinc finger; exon 3 encodes two zinc fingers; exons 4–6 encode one zinc finger each and exon 7 encodes two zinc fingers.

Gene synteny around ZNF450 is conserved in man, mouse, and rat genomes. Upstream is a hypothetical gene encoding an adenylate kinase like-protein containing a YHS domain that may bind metal ions. In the NCBI annotated genome this gene has erroneously been entitled BMP-induced factor 1. Downstream is the NEDD9 gene which encodes a CasL interacting protein, MICAL. All three genes are ordered and transcribed in the same direction. In the ZNF450 promoter immediately upstream of the start of transcription are two promoter elements conserved in rodents and man, a double GC-box that is likely to bind Sp transcription factors and an CACGTG, N-myc binding site. This suggests that ZNF450 may be regulated by the N-myc oncogene.

BTB Domain

A more detailed sequence comparison of those proteins with the most closely related BTB domains to ZNF450 surprisingly shows that not all are zinc finger proteins (Fig. 3). The amino-terminus of murine ZNF450 possesses a BTB domain with 25% identity and 69% similarity to that of the PLZF BTB domain for which the crystal structure has been determined [Ahmad et al., 1998; Li et al., 1999]. The human PLZF BTB domain has been subjected to mutational analysis [Melnick et al., 2000, 2002]. This analysis identified that specific residues that are essential for the dimerisation and transcriptional repression activities. These residues are conserved in ZNF450 suggesting that the ZNF450 BTB domain has a similar structure (Fig. 3). The charged residues D38 and K52 of ZNF450 correspond to residues D35 and R49 of PLZF that on dimerisation form a charged pocket. These charged residues are required for interaction with the co-repressors

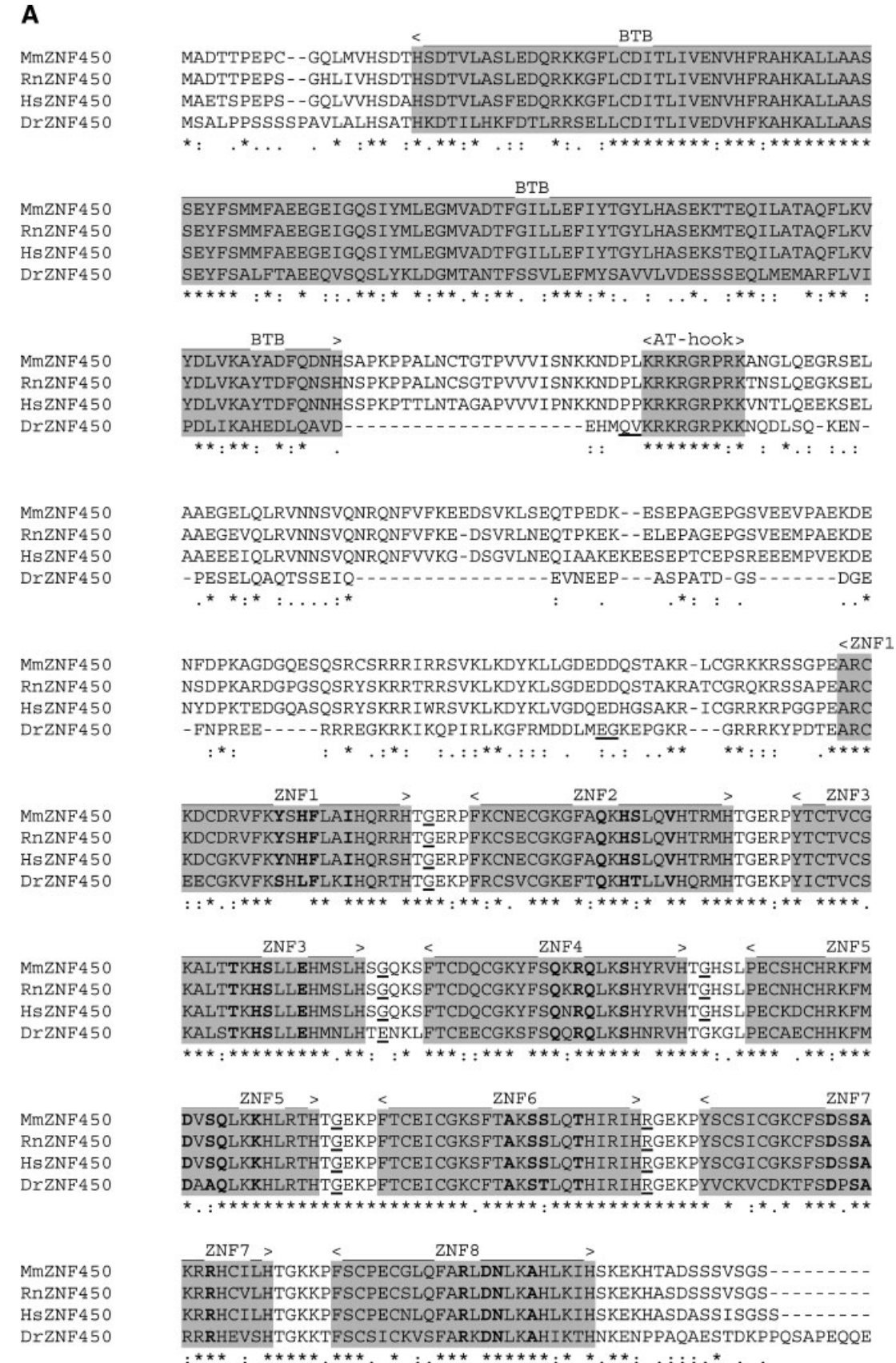
A
BTB
MmZNF450 MADTTPEPC--GQLMVHSDTHSDTVLASLEDQRKKGFLCDITLIVENVHFRAHKALLAAS
RnZNF450 MADTTPEPS--GHLIVHSDTHSDTVLASLEDQRKKGFLCDITLIVENVHFRAHKALLAAS
HsZNF450 MAETSPEPS--GQLVVHSDAHSDTVLASFEDQRKKGFLCDITLIVENVHFRAHKALLAAS
DrZNF450 MSALPPSSSSPAVLALHSATHKDTILHKFDTLRRSELLCDITLIVEDVHFKAHKALLAAS
BTB
MmZNF450 SEYFSMMFAEEGEIGQSIYMLEGMVADTFGILLEFIYTGYLHASEKTTEQILATAQFLKV
RnZNF450 SEYFSMMFAEEGEIGQSIYMLEGMVADTFGILLEFIYTGYLHASEKMTEQILATAQFLKV
HsZNF450 SEYFSMMFAEEGEIGQSIYMLEGMVADTFGILLEFIYTGYLHASEKSTEQILATAQFLKV
DrZNF450 SEYFSALFTAEEQVSQSLYKLDGMTANTFSSVLEFMYSAVVLVDESSSEQLMEMARFLVI
BTB
<AT-hook>
MmZNF450 YDLVKAYADFQDNHSAPKPPALNCTGTPVVVISNKKNDPLKRKRGRPRKANGLQEGRSEL
RnZNF450 YDLVKAYTDFQNSHNSPKPPALNCSGTPVVVISNKKNDPLKRKRGRPRKTNSLQEGKSEL
HsZNF450 YDLVKAYTDFQNNHSSPKPTTLNTAGAPVVVIPNKKNDPPKRKRGRPKKVNTLQEEKSEL
DrZNF450 PDLIKAHEDLQAVD----------EHMQVKRKRGRPKKNQDLSQ-KEN-
<ZNF1
MmZNF450 AAEGELQLRVNNSVQNRQNFVFKEEDSVKLSEQTPEDK--ESEPAGEPGSVEEVPAEKDE
RnZNF450 AAEGEVQLRVNNSVQNRQNFVFKE-DSVRLNEQTPKEK--ELEPAGEPGSVEEMPAEKDE
HsZNF450 AAEEEIQLRVNNSVQNRQNFVVKG-DSGVLNEQIAAKEKEESEPTCEPSREEEMPVEKDE
DrZNF450 -PESELQAQTSSEIQ----------EVNEEP---ASPATD-GS-------DGE
ZNF1 ZNF2 ZNF3
MmZNF450 KDCDRVFKYSHFLAIHQRRHTGERPFKCNECGKGFAQKHSLQVHTRMHTGERPYTCTVCG
RnZNF450 KDCDRVFKYSHFLAIHQRRHTGERPFKCSECGKGFAQKHSLQVHTRMHTGERPYTCTVCS
HsZNF450 KDCGKVFKYNHFLAIHQRSHTGERPFKCNECGKGFAQKHSLQVHTRMHTGERPYTCTVCS
DrZNF450 EECGKVFKSHLFLKIHQRTHTGEKPFRCSVCGKEFTQKHTLLVHQRMHTGEKPYICTVCS
ZNF3 ZNF4 ZNF5
MmZNF450 KALTTKHSLLEHMSLHSGQKSFTCDQCGKYFSQKRQLKSHYRVHTGHSLPECSHCHRKFM
RnZNF450 KALTTKHSLLEHMSLHSGQKSFTCDQCGKYFSQKRQLKSHYRVHTGHSLPECNHCHRKFM
HsZNF450 KALTTKHSLLEHMSLHSGQKSFTCDQCGKYFSQNRQLKSHYRVHTGHSLPECKDCHRKFM
DrZNF450 KALSTKHSLLEHMNLHTENKLFTCEECGKSFSQQRQLKSHNRVHTGKGLPECAECHHKFM
ZNF5 ZNF6 ZNF7
MmZNF450 DVSQLKKHLRTHTGEKPFTCEICGKSFTAKSSLQTHIRIHRGEKPYSCSICGKCFSDSSA
RnZNF450 DVSQLKKHLRTHTGEKPFTCEICGKSFTAKSSLQTHIRIHRGEKPYSCSICGKCFSDSSA
HsZNF450 DVSQLKKHLRTHTGEKPFTCEICGKSFTAKSSLQTHIRIHRGEKPYSCGICGKSFSDSSA
DrZNF450 DAAQLKKHLRTHTGEKPFTCEICGKCFTAKSTLQTHIRIHRGEKPYVCKVCDKTFSDPSA
ZNF7 ZNF8
MmZNF450 KRRHCILHTGKKPFSCPECGLQFARLDNLKAHLKIHSKEKHTADSSSVSGS---------
RnZNF450 KRRHCVLHTGKKPFSCPECSLQFARLDNLKAHLKIHSKEKHASDSSSVSGS---------
HsZNF450 KRRHCILHTGKKPFSCPECNLQFARLDNLKAHLKIHSKEKHASDASSISGSS--------
DrZNF450 RRRHEVSHTGKKTFSCSICKVSFARKDNLKAHIKTHNKENPPAQAESTDKPPQSAPEQQE

Fig. 1.

Fig. 1. (*Continued*)

N-CoR and SMRT and histone deacetylases suggesting that ZNF450 may also dimerise and act as a transcriptional repressor through interaction with co-repressors.

AT-Hook Domain

ZNF450 has an AT-hook motif located between the BTB and zinc finger domain in an otherwise low complexity region (Fig. 1A). The AT-hook motif was first described in high mobility group proteins that are components of chromatin where they preferentially bind to the minor groove of AT-rich regions in double-stranded DNA [Reeves and Nissen, 1990]. The AT-hook motif in ZNF450 is a positively charged stretch of nine amino acids. The arginine residues in the central arginine-glycine-arginine-proline (RGRP) peptide penetrate deep into the DNA helix and interact with the bases of DNA centred on the sequence AA(T/A)T [Maher and Nathans, 1996]. The AT-hook motif in ZNF450 has symmetry around the central glycine residue, KRkRgRpRK, with the Asx hooks at its amino and carboxy-termini likely to play an additional role in DNA binding [Aravind and Landsman, 1998].

Zinc Finger Domain

The ZNF450 protein is predicted to contain eight zinc finger structures of the classical Cys2-

Fig. 1. Protein sequence comparison of the murine ZNF450 isoforms with those of other species. **A**: Comparison of the long ZNF450 isoform with the human, rat, and zebrafish homologues. The amino-terminal BTB/POZ domain is indicated, as is the AT-hook domain and eight C2H2 zinc fingers, ZNF1-8. The carboxy-terminal VITLSKE domain is overlined. Those residues likely to contact DNA at positions −1, +2, +3, and +6 with respect to the alpha-helix of the zinc fingers are shown in bold. The location of the exon/exon boundaries are shown on the protein sequences as underlined residues. Conserved residues are shown by an (*), strongly conserved residues by (:) and weakly conserved residues by (.). A hash (#) indicates a stop codon. **B**: ZNF450 protein sequences showing the comparison of the carboxy-terminal region of the short isoforms from mouse, rat, and man. The short protein isoforms are generated by reading through the donor splice site of exon 2 into intron 2. The abbreviations are: Mm, *Mus musculus*; Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Dr, *Danio rerio*.

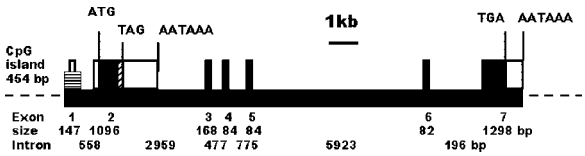

Fig. 2. The gene structure of murine ZNF450. It has seven exons. The ORF of the long protein isoform is indicated by closed boxes and the additional ORF of the short protein isoform by diagonal lines. The CpG island that encompasses exon 1 is indicated by horizontal lines. The initiation methionine codon ATG is shown. The two stop codons corresponding to the short and long protein isoforms, TAG and TGA, are indicated and the potential polyadenylation signals are also shown. The sizes of the exons and introns are indicated.

His2 type where the conserved cysteine residues in two short beta-sheets and the histidine residues in an alpha-helix tetrahedrally coordinate a zinc ion [Pavletich and Pabo, 1991; Narayan et al., 1997]. The amino terminal part of the helix binds the major groove in DNA binding zinc fingers. The five residue linker sequence between fingers has the highly conserved consensus sequence TGEKP. Although a consensus DNA-binding site for ZNF450 has yet to be determined the Zn finger structure indicates it is likely to bind a 24 bp sequence, more than enough to specify a unique location in the genome. In terms of those residues that are likely to contact one strand of DNA at positions −1, +3, and +6 with respect to the alpha-helix, all eight fingers of ZNF450 have no matches in the current databases, suggesting that ZNF450 has the capability to uniquely target specific sites in gene promoters. Those residues that are thought likely to contact DNA are conserved between human and rodent proteins. However, in the zebrafish only zinc fingers 3, 4, 5, 7, and 8 retain all those residues seen in mammals (Fig. 1A) suggesting that they are the most important for determining the sequence specificity of ZNF450.

VITLSKE Domain

The Zn fingers are separated from the carboxy-terminus of the protein by a region with a net charge of −10. Close to the carboxy-terminus is a novel highly conserved 22-residue domain located in an otherwise low homology region (Fig. 1A). This 22-residue domain is unique to ZNF450 homologous proteins. The conserved core residues, VITLSKE, are located in the centre of a predicted beta-sheet, turn, alpha-helix secondary structure. Beyond the VITLSKE domain, the carboxy-terminal is very variable in length and sequence composition suggesting that it does not have a conserved function.

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                          *   :*   .. *  . *** .. *   .* **: :**. *  *      :
MmZNF100    1        MDFPQHSQRVLEQLNQQRQLGLLCDCTFVVDGVDFKAHKAVLAACSEYFKMLFVDQ---
MmZNF238    1        MEFPDHSRHLLQCLSEQRHQGFLCDCTVLVGDAQFRAHRAVLASCSMYFHLFYKDQLDK
MmBach2     9   SPMYVYESTVHCANILLGLNDQRKKDILCDVTLIVERKEFRAHRAVLAACSEYFWQALVGQTKD
MmC3IP1     5   MAPKDIMTNTHAKSILNSMNSLRKSNTLCDVTLRVEQKDFPAHRIVLAACSDYFCAMFTSELSE
MmZNF450    9   CGQLMVHSDTHSDTVLASLEDQRKKGFLCDITLIVENVHFRAHKALLAASSEYFSMMFAEEGEI
HsPlzf      6   MGMIQLQNPSHPTGLLCKANQMRLAGTLCDVVIMVDSQEFHAHRTVLACTSKMFEILFHRN---
HsPlzf_SS                     alpha1           beta1 beta2 alpha2   alpha3
Mutations                      X            -             +

                              :  :*:: *   :  .     ::: * *.:  :
MmZFP100       KDVVHL--DISNAAGLGQVLEFMYTAKLSLSPENVDDVLAVASFLQMQDIVTACHTLKSLAE 116
MmZFP238       RDIVHLNSDIVTAPAFALLLEFMYEGKLQFKDLPIEDVLAAASYLHMYDIVKVCKKKLKEKA 121
MmBach2        DLVVSL-PEEVTARGFGPLLQFAYTAKLLLSRENIREVIRCAEFLRMHNLEDSCFSFLQTQL 133
MmC3IP1        KGKPYVDIQGLTAATMEILLDFVYTETVHVTVENVQELLPAACLLQLKGVKQACCEFLESQL 130
MmZNF450       GQSIYMLEGMVAD-TFGILLEFIYTGYLHASEKTTEQILATAQFLKVYDLVKAYADFQDNHS 133
HsPlzf         --SQHYTLDFLSPKTFQQILEYAYTATLQAKAEDLDDLLYAAEILEIEYLEEQCLKMLETIQ 126
HsPlzf_SS        Beta3       alpha4                 alpha5        alpha6
Mutations                          X                 X
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Fig. 3. Comparision of sequence homology of the mouse ZNF450 BTB domain with closely related mouse proteins and the crystal secondary structure of the human Plzf. The proteins are mouse ZNF100/Polyomavirus late initiator promoter binding protein [Schulz et al., 1995]; ZNF238/RP58 [Meng et al., 2000]; Bach2, a basic leucine zipper transcription factor [Oyake et al., 1996]; C3IP1, a kelch-like protein (accession number NP_694768) and PLZF [Ahmad et al., 1998; Li et al., 1999]. Conserved residues are shown by an (*), strongly conserved residues by (:) and weakly conserved residues by (.). Residues conserved in two or more sequences are shown in bold. Secondary structure of the human PLZF is shown, HsPLZF_SS. Those residues of human PLZF that on mutation disrupt dimerisation [Melnick et al., 2000, 2002] are indicated by X and are conserved in ZNF450. The residues D35 and R49 of PLZF that on dimerisation form a charged pocket are also highly conserved in ZNF450 and are indicated by (−) and (+).

BMP-2 Regulation of ZNF450 mRNA Expression in Cell Lines

ZNF450 mRNA expression was measured by RT-real time PCR. Serum-starved levels of ZNF450 mRNA expression were compared in four murine cell lines (Fig. 4A). The highest level of expression was found in NIH-3T3 fibroblasts, with moderate levels of expression found in the C3H10T1/2 pluripotent embryonic and the C2C12 myoblast cell lines. Expression was comparatively low in MC3T3-E1 preosteoblasts.

ZNF450 mRNA was induced after 4 h and was upregulated three to fivefold after 24 h in C3H10T1/2, C2C12, and NIH-3T3 fibroblastic cells when stimulated with 100 ng/ml BMP-2 $P < 0.05$ (Fig. 4B–D). In C3H10T1/2 cells, a dose response showed that there was no increase in expression with 30 ng/ml BMP-2 treatment after 24 h whereas 100 and 300 ng/ml both upregulated ZNF450 (Fig. 4E). A similar trend was observed in MC3T3-E1 cells, in both time and dose response experiments, but owing to the low levels of ZNF450 expression in this cell line they were not statistically significant (data not shown).

Treatment with the reversible protein synthesis inhibitor cycloheximide in C3H10T1/2 cells largely prevented the 100 ng/ml BMP-2 induction of ZNF450 after 24 h as determined by northern blot analysis normalised to the expression of the housekeeping gene *G3PDH*. Densitometric analysis showed that BMP-2 upregulated ZNF450 104%-fold after 24 h in the absence of cycloheximide, but in the presence of cycloheximide this increase in expression was reduced to only a 13% increase. Together with the temporal pattern of expression these data suggest that ZNF450 may not be directly regulated by BMP-2 and can be termed an intermediate responsive gene.

In C2C12 cells we examined the time courses of BMP-2 induction of ID1, Runx2, and Sp7. ID1 was rapidly induced reaching a peak after 1 h and subsequently declining to basal levels (Fig. 5A). The time course of BMP-2 induction of Runx2 and Sp7 was similar to that of ZNF450 being significantly induced after 4 h (Fig. 5B,C). The greatest extent of the inductions varied, with Sp7 being upregulated over 1,200% and ID1 over 600%. The induction of Runx2 was more than 300% after 24 h, similar to that of ZNF450 (Fig. 4B). For experiments using C2C12 cells, expression data was normalised to beta-actin. Data normalised to the housekeeping gene *G3PDH* gave similar results (data not shown), indicating that changes in the levels of expression of the genes of interest occurred and that expression of the control genes remained essentially constant.

Tissue Distribution of ZNF450 mRNA

The expression of ZNF450 mRNA in adult tissues was examined by RNA blotting and RT-real time PCR (Fig. 6). Using an exon 1-2 cDNA probe that detects both isoforms a major transcript of approximately 3 kb was found (Fig. 6A) being highest in liver, testis, and kidney and low in brain and skin. Since the expected mRNA lengths analysis (2.8 and 2.5 kb for the long and short protein isoforms, respectively) of both isoforms are not readily resolved by RNA blotting we also examined the tissue distribution by RT-real time PCR using isoform specific primers relative to the expression of the housekeeping gene, β-actin (Fig. 6B,C). Using the more sensitive RT-PCR method both isoforms were detected in all tissues examined. The expression of the long protein isoform was highest in testis and kidney and that of the short protein isoform was highest in liver and testis and low in bone marrow. Generally, in the tissues the mRNA for the long protein isoform was predominantly expressed being approximately threefold more abundant than the short protein isoform (assuming equal rates of PCR amplification for the two isoforms). However, in liver and spleen the expression of the isoforms were similar. The relative abundance of the two isoforms of ZNF450 mRNA was examined by RT-real time PCR in C3H10T1/2, C2C12, and NIH-3T3 fibroblast cell lines using a common sense primer (Fig. 7). The mRNA for the full length protein was approximately six times more abundant than that of the short protein isoform. There were no significant differences between the cell lines.

DISCUSSION

In pre-chondrocytic and osteoblast-like cells BMP-2 has been shown to upregulate a number of transcription factors such as Sp7/Osterix and Runx2 that are involved in regulating these differentiation pathways [Ducy et al., 1997; Lee et al., 1999; Nakashima et al., 2002]. We have identified a novel murine gene, *ZNF450*, that was differentially expressed in

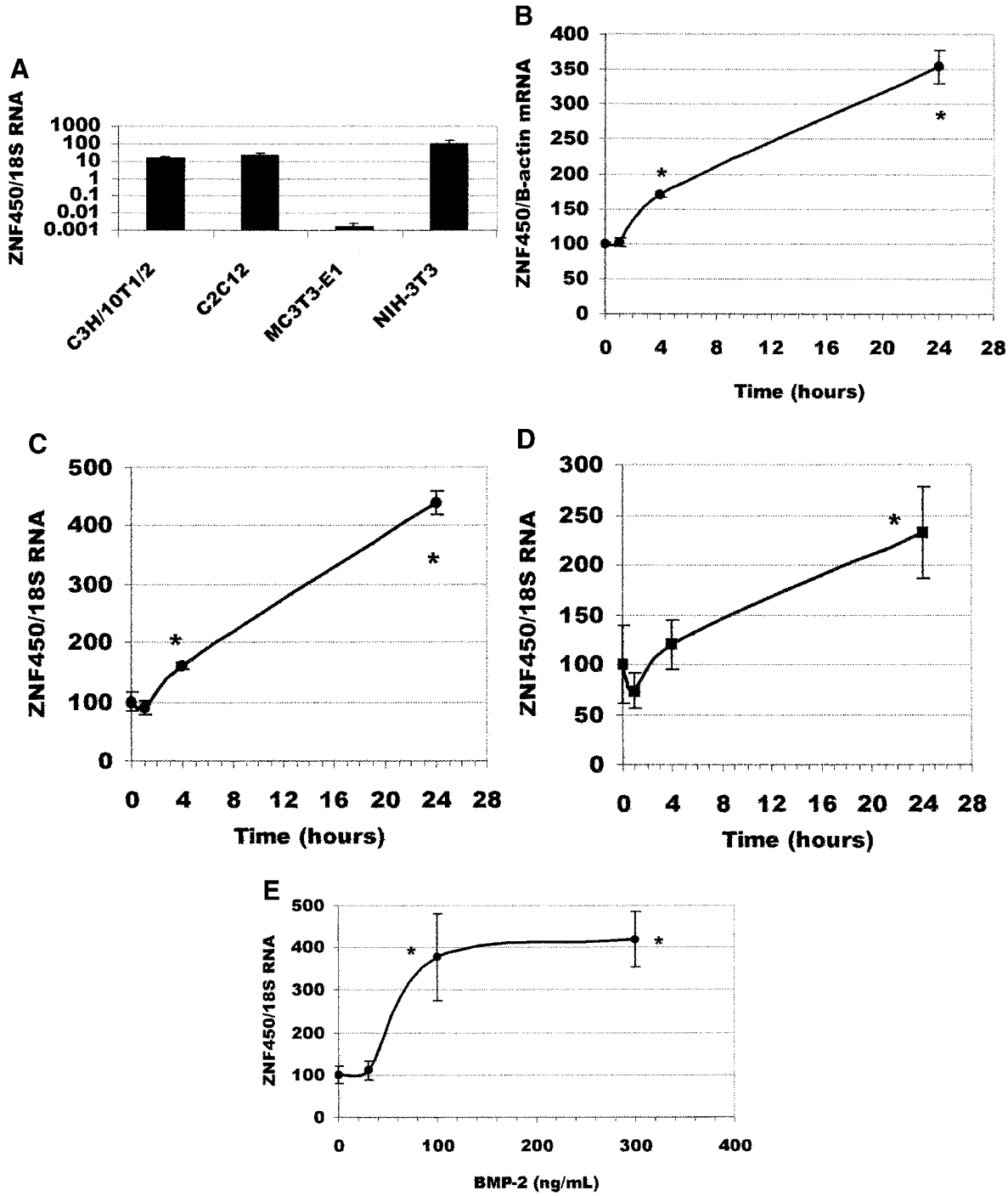

Fig. 4. ZNF450 mRNA expression in cell lines determined by RT-real time PCR. **A**: Basal levels of ZNF450 mRNA expression in unstimulated cell lines relative to 18S RNA. **B**–**D**: Time course of the BMP-2 effect on ZNF450 mRNA expression. The levels of ZNF450 mRNA expression were examined after 0, 1, 4 and 24 h treatment with 100 ng/ml BMP-2. The cell lines were: C2C12 (B), C3H10T1/2 (C), and NIH-3T3 fibroblasts (D) and were normalised to beta-actin mRNA (B) or 18S RNA (C and D). Statistically significant increases in ZNF450 mRNA expression relative to control are indicated with asterisks $P < 0.05$. **E**: Dose response in C3H10T1/2 cells. C3H10T1/2 cells were treated with 30, 100, 300 ng/ml BMP-2 for 24 h.

the pluripotent C3H10T1/2 cell line treated with BMP-2. The gene encodes a 79 kDa transcription factor that has an amino-terminal BTB domain and eight C2H2 zinc fingers towards the carboxy-terminus. In C2C12 cells the time course of ZNF450 induction is similar to that of Runx2 and Sp7 being upregulated after 4 h. In comparison ID1, is rapidly and transiently

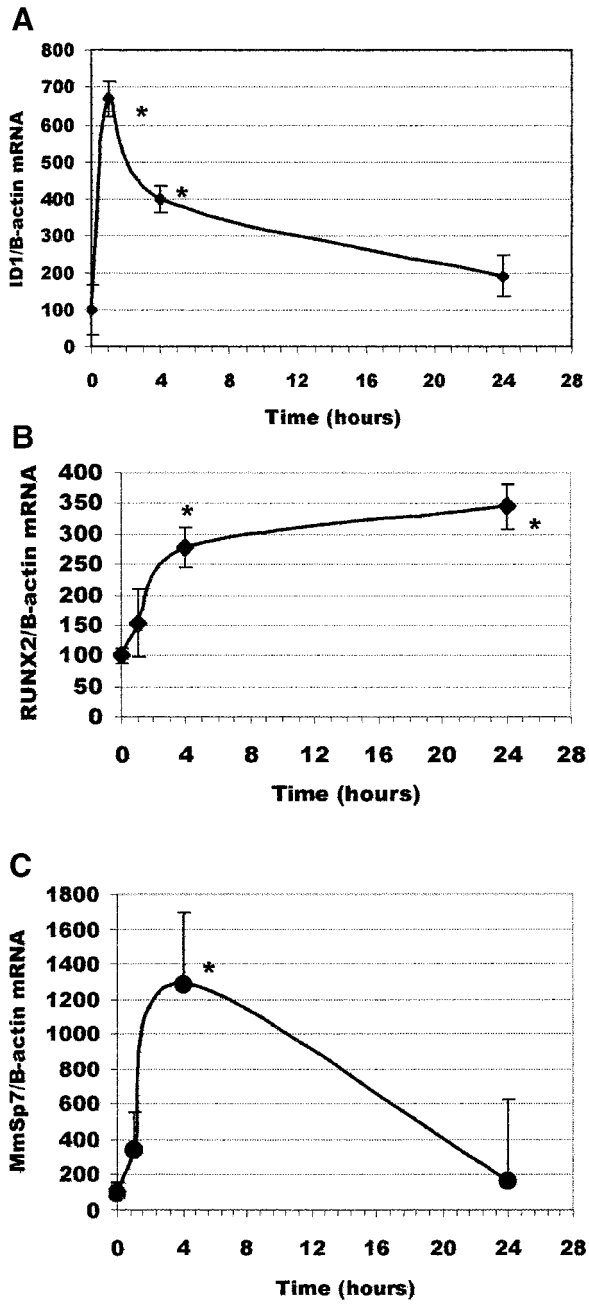

Fig. 5. Time course of effect of BMP-2 treatment on C2C12 cells. Messenger RNA expression was determined by RT-real time PCR. The levels of ID1 (**A**), Runx2 (**B**), and Sp7/osterix (**C**) mRNA expression were examined after 0, 1, 4, and 24 h treatment with 100 ng/ml BMP-2. Statistically significant increases in ID1 expression were seen at 1 and 4 h; for Runx2 at 4 and 24 h, and for Sp7 at 4 h ($P < 0.05$).

induced by BMP-2 during the transdifferentiation of C2C12 myoblasts into osteoblasts [Katagiri et al., 1994] and is termed an early responsive gene. BMP-responsive elements have been identified in the ID1 promoter [Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002], but similar motifs are not present in the ZNF450 promoter. Gene expression microarray profiling experiments have been used to identify BMP-2 regulated genes involved in mesenchymal differentiation [Vaes et al., 2002; Balint et al., 2003; Clancy et al., 2003; De Jong et al., 2004]. As far as we can ascertain ZNF450 has not been described previously as a BMP-2 responsive gene by these profiling experiments.

C3H10T1/2 cells can be induced to differentiate towards an osteoblastic phenotype by BMP-2 and BMP-2 upregulates ZNF450 in these cells. BMP-2 also induced ZNF450 in NIH3T3 embryonic fibroblasts that are generally considered a non-osteoblastic cell line since they do not produce a mineralised matrix [Selvamurugan et al., 2000]. However, NIH3T3 cells can be induced to express alkaline phosphatase and osteocalcin, two markers of osteoblastic differentiation [Shui and Scutt, 2002] suggesting that these embryo-derived cells possess some characteristics of osteoblasts. The dose of BMP-2 used to treat C3H10T1/2 directs their pattern of differentiation with low doses, 25–50 ng/ml favouring adipocytic differentiation and high

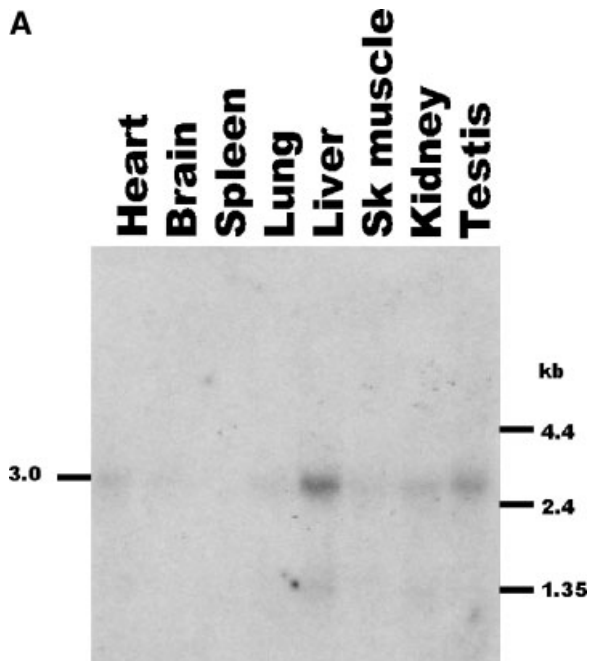

Fig. 6. Tissue distribution of ZNF450 mRNA. **A**: Northern blot of adult mouse tissues probed with an cDNA exon 2 probe common to both isoforms. A 3 kb major transcript is indicated. RT-real time PCR determination of expression levels of long (**B**) and short (**C**) protein isoform of ZNF450 mRNA compared to beta-actin in adult mouse tissues. Note the high level of expression of ZNF450 in skeletal muscle determined by PCR is a consequence of the low level of β-actin in this tissue.

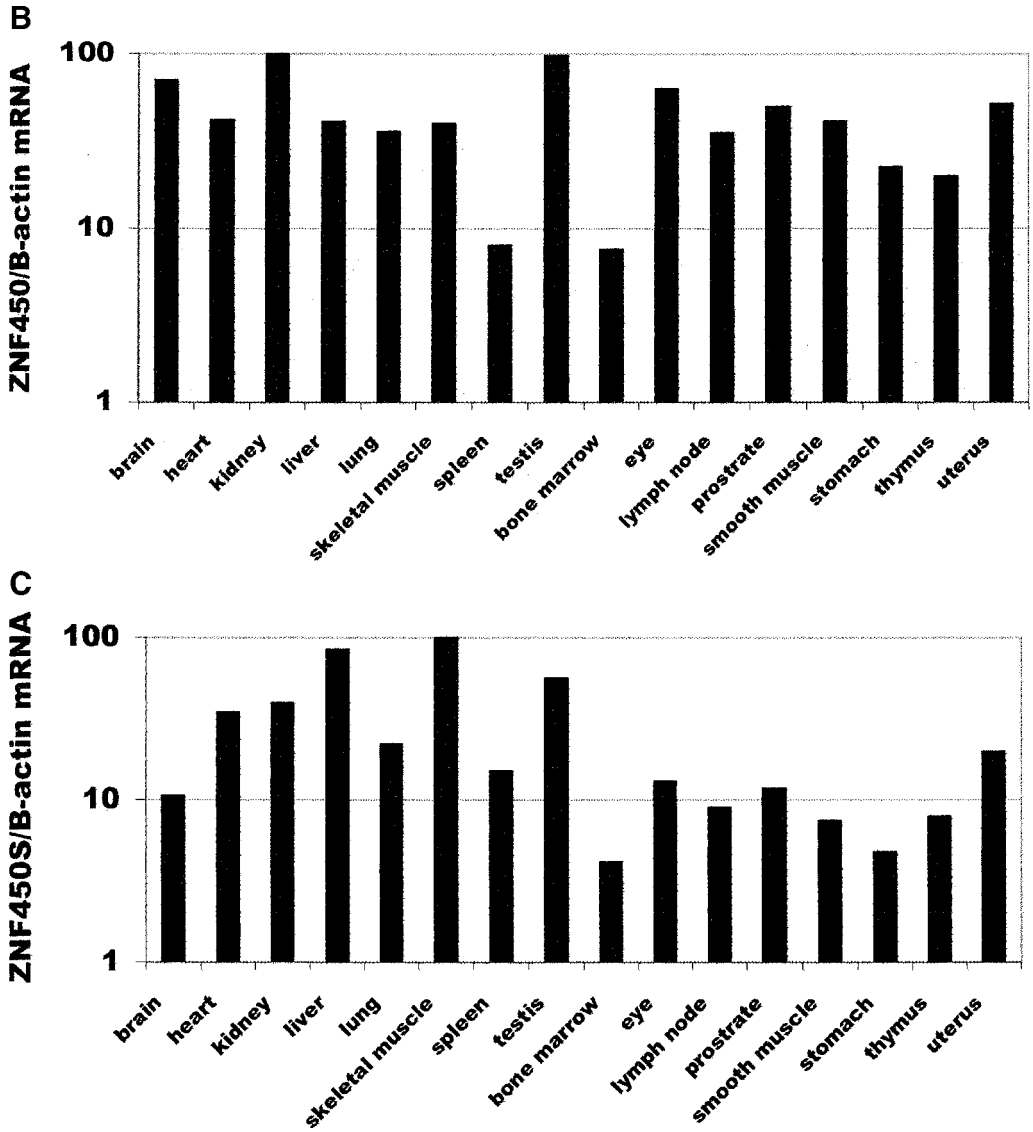

Fig. 6. (*Continued*)

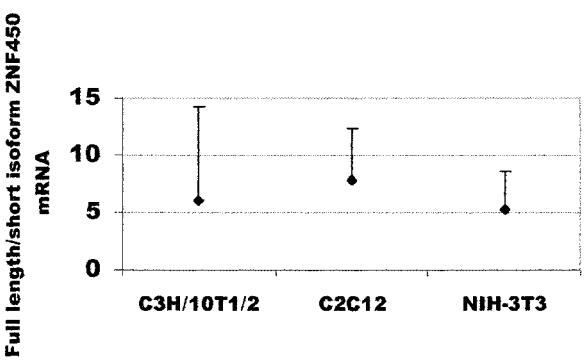

Fig. 7. RT-real time PCR determination of the expression ratio of the short to the long protein isoform of ZNF450 mRNA in unstimulated mouse cell lines. The mRNA for the long protein isoform is 5–7 times more abundant than that of the short protein isoform.

doses, 100–1,000 ng/ml favouring chondrocytic and osteoblastic differentiation [Wang et al., 1993; Puleo, 1997; Shea et al., 2003]. We found that treatment with 100 and 300 ng/ml BMP-2 induced ZNF450 expression, but 30 ng/ml did not induce expression. Generally, genes that exhibit changes in their expression level within 1–4 h of BMP-2 treatment are involved in the activation of osteoblastic or repression of non-osteoblastic developmental pathways [Balint et al., 2003; De Jong et al., 2004]. Although our data on ZNF450 upregulation by BMP-2 does not provide direct evidence that ZNF450 plays a role in mesenchymal cell-osteoblast differentiation the time course and concentration dependence of ZNF450 induction support the idea that it has a potential

significance role in mesenchymal differentiation to a chondrocytic/osteoblastic linage.

Although murine ZNF450 was cloned from a testis cDNA library, its expression is widespread, being found in all tissues examined. However, the level varied more than 10-fold, being highest in liver, testis, and kidney, suggesting that ZNF450 plays an important transcriptional role in these organs. The human ZNF450 cDNA was isolated from a brain cDNA library and RT-PCR analysis showed that human ZNF450 mRNA was expressed in placenta, lung, liver, kidney, thymus, prostate, ovary, and small intestine [Ishikawa et al., 1997]. However, it was below the limit of detection in brain, heart, skeletal muscle, pancreas, spleen, and testis indicating that there are differences in tissue expression between man and mouse.

Although there may be as many as 800 zinc finger transcription factors in the mouse genome only 42 also have a BTB domain and none are closely related to ZNF450. Two BTB-8 fingered transcription factors, myoneurin and MAZR/ZNF278 have low pairwise sequence identities at the protein level (26 and 22%, respectively) and similar arrangements of their protein domains to ZNF450, however, all three genes have different exon structures suggesting that they are only distantly related. The function of ZNF450 remains to be determined, but it may be a transcriptional repressor since other BTB-zinc finger transcription factors such as Tramtrack, PLZF, BCL-6, and BAZF are transcriptional repressors [Huynh and Bardwell, 1998; Okabe et al., 1998; Badenhorst et al., 2002; Melnick et al., 2002]. They mediate transcription repression through their BTB domain. BTB domains usually form homomeric dimers, but can form heteromeric dimers [Bardwell and Treisman, 1994; Widom et al., 2001; Pagans et al., 2002].

ZNF450 has an AT-hook motif located between the BTB and ZNF domains. This AT-hook motif is also found in MAZR. The AT-hook motif does bind to the minor groove of AT-rich regions [Reeves and Nissen, 1990] and the BTB domain-containing bric-a-brac genes, *bab1* and *bab2*, from *Drosophila* contain an AT-hook motif and a pipsqueak domain that together are required for binding to regions containing TA or TAA repeats [Lours et al., 2003]. The presence of an AT-hook motif in ZNF450 suggests that it also targets AT-rich regions such as those found in TATA boxes, scaffold-associated and matrix associated regions on chromosomes, in addition to that provided by the sequence specificity encoded by the zinc fingers.

We have identified two ubiquitously expressed mRNA isoforms of ZNF450, one encoding a full-length protein and the other a carboxy-terminal-truncated protein. The long protein isoform is the predominant isoform in the cell lines examined, however, there were tissue specific differences in the expression of the two isoforms suggesting that the relative abundance of the two isoforms may have a role in tissue function. The short isoform is found in man and rodents suggesting that the splicing mechanism regulating its expression is conserved. Since the short protein isoform has only one zinc finger it would be expected to have a low affinity for DNA.

The transcriptional mechanism by which the ZNF450 short protein isoform is formed is not unique. Another mouse BTB-ZNF protein, ZNF509, has two protein isoforms, a long protein isoform that has seven zinc fingers and a short protein isoform that has one zinc finger (accession nos. XM_132091 and BAC32172, respectively). The mRNA of the short isoform of ZNF509 is generated by extending transcription from exon 2 into intron 2, in an identical manner to that of the ZNF450 short protein isoform. To date, ZNF450 and ZNF509 short protein isoforms are the only mammalian BTB-ZNF proteins with one zinc finger, although nine human BTB-ZNF proteins have just two. However, in *Drosophila*, the BTB-ZNF protein, GAGA factor has only one zinc finger and binds genomic DNA as a dimer [Benyajati et al., 1997], which supports the idea that the ZNF450 short protein isoform may also bind DNA as a dimer.

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