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RESEARCH ARTICLE

Mapping the transcription repressive domain in the highly conserved human gene *hnulp1*

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Abstract HNULP1, a new member of the basic helix-loop-helix transcription factors, contains a DUF654 domain in its C-terminus and is highly conserved from *Drosophilae*, yeast, zebrafish to mouse. The function of this motif, however, is currently unknown. In this research, we fused five deletion fragments of the DUF654 domain to the GAL4 DNA-binding domain and then co-transfected with plasmids L8G5-Luc and VP-16. The analysis of the GAL4 luciferase reporter gene indicated that fragments from 228 to 407 amino acids in the DUF654 domain had a strong transcription repression activity. Therefore, this study lays a solid foundation for research on the mechanism of *hnulp1* transcriptional regulation and the function of the DUF654 domain.

Keywords DUF654, luciferase reporter gene, transcriptional activity, *hnulp1*

1 Introduction

Transcription factors play an important role in cell life. Basic helix-loop-helix (bHLH) is a special category of transcription factors. This protein family is identified by a highly conserved bHLH domain for DNA binding and protein-protein interaction. It is about 60 amino acids length and contains a basic DNA-binding region like E-box which can recognize six conserved nucleotide sequences in the N-terminus of about 15 amino acids. Moreover, it has two α helices of about 15–16 amino acids, containing several conserved amino acids that are separated by a variable loop region (HLH) (Murre et al., 1989).

HNULP1 is a new member of the basic helix-loop-helix transcription factors. Previous studies demonstrated that *nulp1* is expressed broadly in both early mouse embryonic and adult tissues and especially with a higher level in the brain. Nuclear localization sequence in the N-terminus shows the NULP1 protein distributed in the nuclei (Olsson et al., 2002). Our study shows that the human gene *hnulp1* expressed at a relatively higher level in the adult heart. It is highly conserved from *Drosophilae*, yeast, zebrafish, to mouse. The analysis of the Gal-4 luciferase reporter gene indicated that HNULP1 functions as a transcriptional repressor of serum response factor (SRF) (Cai et al., 2006). The human gene *hnulp1* contains a DUF654 domain in its C-terminus which is a conserved and very large motif spanning about 400 residues. The sequences of DUF654 are rich in polar and hydrophobic amino acids. Several nearly invariant residues (lysine, praline, alanine, and tyrosine) at specific positions were also identified in this region. The function of this motif, however, is currently unknown. Analysis of the L8G5-Luc reporter gene indicated that HNULP1 was a strong transcriptional repressive factor in which the DUF654 motif represented basal transcriptional repressive activity (Cai et al., 2006).

To further study mechanisms of *hnulp1* transcriptional regulation and of DUF654 domain transcription suppression, a mammalian transcription activation system was constructed. Five deletion fragments of the DUF654 domain were fused to the Gal4 DNA-binding domain and then transfected into COS-7 cells. Forty-eight hours later, luciferase activity was assayed. Analysis of the L8G5-Luc reporter gene mapped the transcription repressive domain in the DUF654 domain.

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2 Methods

2.1 Plasmid construction

Δ228–289, Δ228–343, Δ228–407, Δ440–568, Δ569–676 amino acids of the DUF654 cDNA sequence were amplified by PCR using human heart cDNA as the template with 5 pairs of primers P1/P2, P3/P4, P5/P6, P7/P8, P9/P10, respectively (Table 1). The PCR procedure was as follows: pre-denaturation at 94°C for 4 min, followed by denaturation at 94°C for 4 min, annealing at 60°C for 30 seconds, polymerization at 72°C for 2 min, 35 cycles and then, polymerization at 72°C for 8 min.

PCR products Δ228–289aa, Δ228–343aa, Δ228–407aa, Δ440–568aa were cloned into the *Eco*RI and *Sal*I sites in vector pCMV-BD. PCR product Δ569–676aa was cloned into the *Eco*RI and *Bam*HI sites in vector pCMV-BD. The set of the constructed plasmids were named pCMV-BDΔ228–289, pCMV-BDΔ228–343, pCMV-BDΔ228–407, pCMV-BDΔ440–568 and pCMV-BDΔ569–676.

2.2 Cell culture and transient transfection

COS-7 cells were cultured and maintained according to the standard methods in DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2. Twenty-four hours later, with the use of So-fast™ (Xiamen Sunma Biotechnology Co., Ltd.), COS-7 cells were transfected with plasmids pCMV-BD, pCMV-BDΔ228–289, pCMV-BDΔ228–343, pCMV-BDΔ228–407, pCMV-BDΔ440–568, pCMV-BDΔ569–676, pL8G5-Luc and VP-16, respectively. According to the method described previously (Liu et al., 2005), the transfected cells were treated as follows: DNA was dissolved by 50 μL DMEM. At the same time, 2 μL So-fast™ was dissolved by 50 μL DMEM and incubated at room temperature for 5 min, then mixed with DNA-DMEM. The 100 μL mixture was added into a 24-well plate to cover the cells and were then revolved gently to mix and incubated at 37°C for cell culture.

2.3 Luciferase and β-galactosidase assays

The luciferase activity assay was performed according to the established protocols (Promega luciferase assay system). Forty-eight hours after transfection, the media was removed from the culture plate well and the cells were washed once with PBS. The cells were covered by 80 μL lysis buffer and incubated at room temperature for 15 min, followed by 80°C for 10 min and 37°C for 10–15 minutes. After incubation, the cells were scraped into microcentrifuge tubes and centrifuged for 5 minutes at 12000 × g for 10 min at 4°C. Then, the supernatant was transferred to a new microcentrifuge tube and immediately the supernatant was assayed for luciferase activity. In the assay, ONPG (20 μL 0.4%) was incubated with 20 μL of supernatant at 37°C for 30 min to measure β-galactosidase activity. The reaction was quenched by the addition of 50 μL of 1 mol/L Tris-base and the transfection efficiency at 420 nm was investigated with a spectrophotometer. Enzyme amounts were calculated by using transfection efficiency.

2.4 Transcription repression activity assay

Transcription repression activity of the fusion protein is the ability of transcription repression by the GAL4-Luc reporter gene. In each assay, cells were transfected with the constructed plasmids, GAL4-Luc reporter gene plasmid and β-galactosidase expression plasmid. Transcription repression activity was represented by the ratio of luciferase activity and β-galactosidase activity in the same well. Data averages were calculated after normalization for β-galactosidase activity. A relative light unit (RLU) was used to represent luciferase activity and is the ratio between light emission and of β-galactosidase activity.

2.5 Statistical analysis

Variations in luciferase activity were measured by variance analysis.

Table 1 Sets of specific oligonucleotide primers used in the study

primer	orientation	nucleotide sequence	fragments size/bp
P1	sense	5′-ACCGAATTCCGCTACAGCAAACCAGGTCTG-3′	
P2	antisense	5′-GTCGACTCAGAGTGAGTCAACGTGGTAAGG-3′	204
P3	sense	5′-ACCGAATTCCGCTACAGCAAACCAGGTCTG-3′	
P4	antisense	5′-GTCGACTCACAGGTAGAAGCTCCTGTTCTC-3′	372
P5	sense	5′-ACCGAATTCCGCTACAGCAAACCAGGTCTG-3′	
P6	antisense	5′-GTCGACTCACTCCCACTCCTGGAAGAGGC-3′	558
P7	sense	5′-GAATTCCAGAGCTCTGCCAGGCAGAAGGC-3′	
P8	antisense	5′-GTCGACTCAGACGGCTTCCTTGATCTCAG-3′	402
P9	sense	5′-GGATCCGCTGCCCTGCCCCCGGAC-3′	
P10	antisense	5′-TCGGAATTCTTTTCGGTCACCTCTGCGGA-3′	365

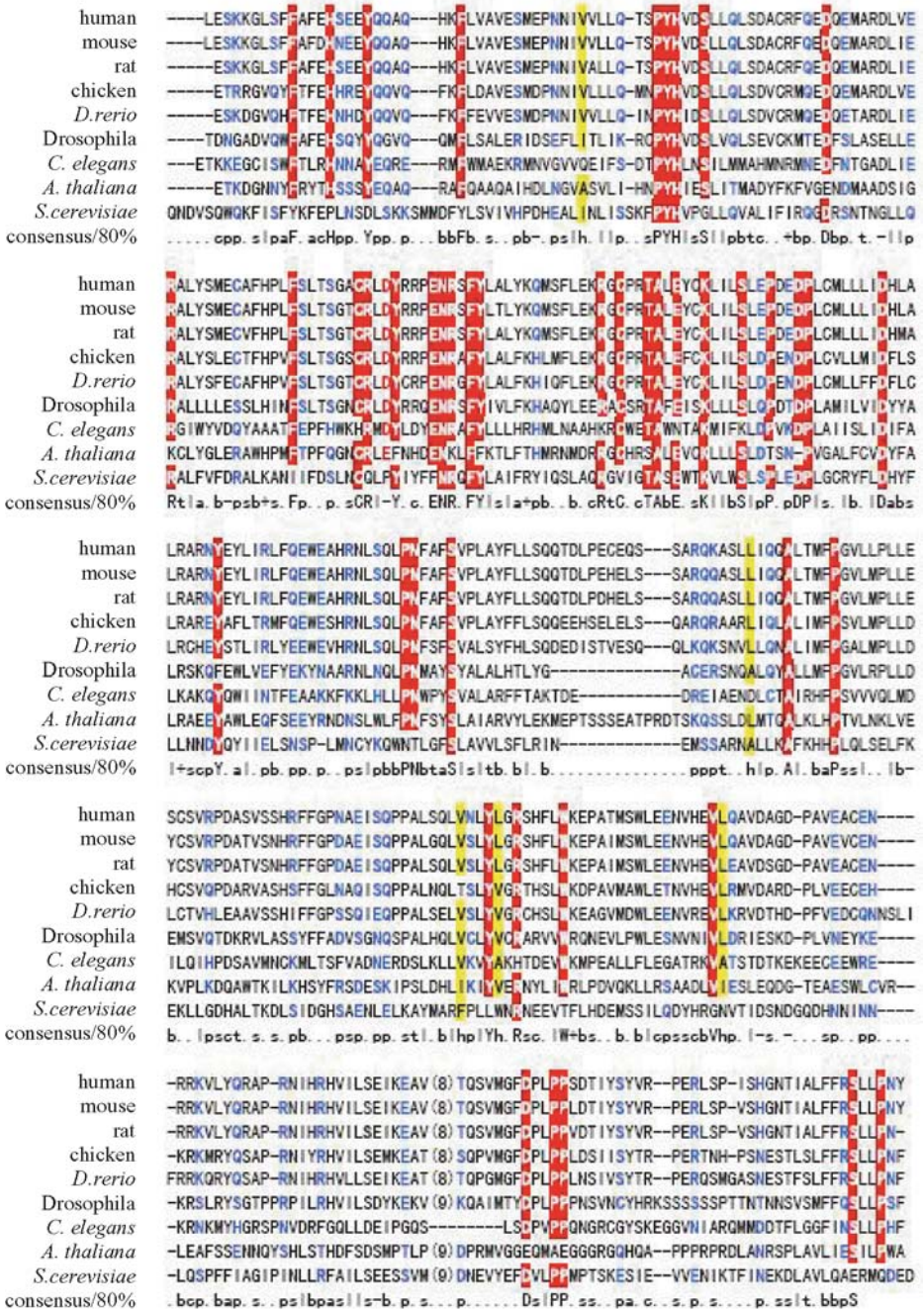

Fig. 1 The DUF654 domain is conserved during evolution. Note: Alignment of DUF654 domain with other orthologues. Highly conserved amino acids are indicated in red. Polar amino acids are indicated in blue. Hydrophobic amino acids are indicated in yellow. Capital letters represent amino acids. Lower-case letters: h, hydrophobic; a, aromatic; b, big; l, aliphatic; p, polar; s, small; t, tiny. c, charged; -, negative; *, Thr/Ser; +, positive. DDBJ/EMBL/GenBank accession No. for the sequences are mouse nulp1 (U94988); rat (XM214707); *Drosophila* (NM139917); *G. gallus* (XM423211); *C. elegans* (NM059946); zebrafish (NM001005399); *S. cerevisiae* (NP010620) and *A. thaliana* (NM130257). The sequence of *G. gallus* is incomplete. The consensus sequence of the domain is calculated and colored using Chroma (Goodstadt and Ponting, 2001).

3 Results

3.1 Bioinformatics analysis

Analysis with the Prosite program (http://www.expasy.ch/prosite) indicated that HUNLP1, a predicted protein with 676 amino acids, contained a bHLH domain and a DUF654 domain in its N-terminal and C-terminal, respectively. A sequence alignment was done using the CLUSTAL W software program (Thompson et al., 1994). Results indicated that HNULP1 was highly conserved from *Drosophilae*, yeast, zebrafish to mouse and

shares 82% identity with mouse, 55% identity with zebrafish, 35% identity with *Drosophilae* and 19% identity with yeast (Fig. 1). Also, the DUF654 domain in the C-terminus was highly conserved which shares a 92.2% identity with mouse, 62.4% identity with zebrafish, 40.7% identity with *Drosophilae* and 23.2% identity with yeast. Moreover, it was a very large motif spanning about 400 residues in the C-terminus region of the proteins. The sequences of DUF654 were rich in polar and hydrophobic amino acids. Several nearly invariant residues (lysine, praline, alanine, and tyrosine) at specific positions were also identified in this region. The function of this motif, however, is currently unknown.

3.2 Plasmid construction of five deletion fragments in the DUF654 domain

Our studies demonstrated that the DUF654 domain had strong transcriptional repressive activity (Cai et al., 2006). To further study the DUF654 domain transcription repression mechanism, five luciferase reporter gene systems were constructed in this study. The basic construction principle was that a sample protein was fused to the DNA binding domain (DBD) of the GAL4 transcription factor. The fusion protein can bind to an upstream regulation sequence of the luciferase reporter gene (GAL4-Luc). If the protein has transcription activation activity, then the luciferase gene is activated, and vice versa. Five deletion fragments of the DUF654 domain were amplified by PCR and cloned into vector pCMV-BD. pCMV-BDΔ228–289, pCMV-BDΔ228–343, pCMV-BDΔ228–407 and pCMV-BDΔ440–568 were digested by the *Eco*RI and *Sal*I, while pCMV-BDΔ569–676 was digested by *Eco*RI and *Bam*HI. Construct correctness was confirmed by electrophoresis. The sequence sizes were 204, 372, 558, 402 and 365 bp, respectively (Fig. 2). Further sequencing demonstrated that the insertion was accurate and the inserted sequences were completely consistent with the *hnulp1* sequence from the GeneBank (GenBank No. DQ321703) without mutations or deletions (Fig. 3).

3.3 Mapping and analysis of DUF654 domain transcription repression activity

COS-7 cells were transfected with recombinant plasmids containing five deletion fragments of the DUF654 domain. Forty-eight hours later, luciferase activity was assayed (Cai et al., 2006). With vector pCMV-BD as a

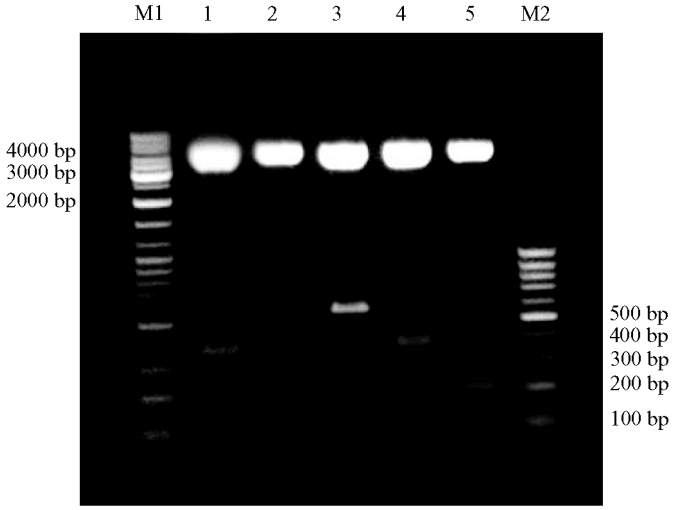

Fig. 2 Restriction analysis of five deletion plasmids of pCMV-BD-DUF654. Note: M_1:10000 bp DNA marker; 1: pCMV-BDΔ569–676; 2: pCMV-BDΔ440–568; 3: pCMV-BDΔ228–407; 4: pCMV-BDΔ228–343; 5: pCMV-BDΔ228–289; M_2: 1000 bp DNA marker.

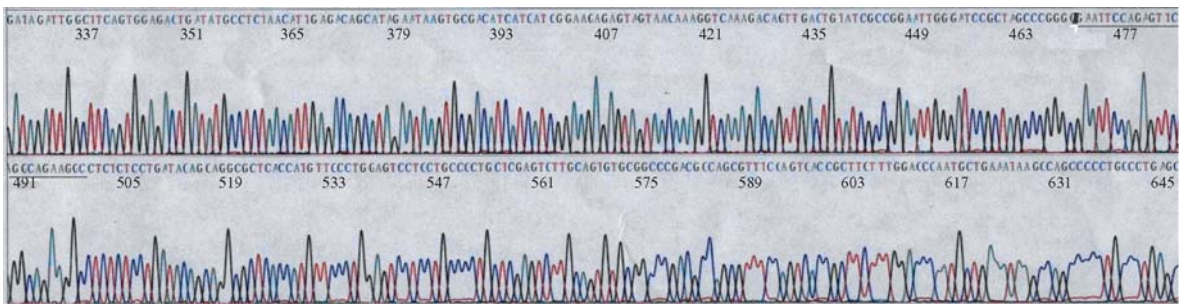

Fig. 3 pCMV-BDΔ440–568 sequencing results. Note: Primer sequence is indicated by a black line (470–568 bp). The left of primer sequence is pCMV-BD sequence, the right is fragments Δ440–568 sequence.

control, deletion fragments of the DUF654 domain from the N-terminal to the C-terminal repressed transcriptional activity by 55.45%, 27.57%, 7.87%, 18.7% and 78.64%. LexA-VP16 plays a role in magnifying the expression efficiency of the luciferase reporter gene. Through adding LexA-VP16, deletion fragments of the DUF654 domain from N-terminal to C-terminal, transcriptional activity was repressed by 30.72%, 10.08%, 2.32%, 5.72% and 89.45%. This study verified that fragments from 228 to 407 amino acids of the DUF654 domain had strong transcriptional repressive activity (Fig. 4).

Discussion

HNULP1 is a new member of basic helix-loop-helix (bHLH) transcription factors. Previous studies demonstrated that the helix-loop-helix region was preceded by a lysine-rich basic region without the core sequence (EXXR). It may represent a new class of bHLH transcription factors (Olsson et al., 2002). Our study indicated that HNULP1 contained a DUF654 domain in its C-terminus. This domain was highly conserved and was a very large motif spanning about 400 residues in the C-terminus region of proteins. The sequences of DUF654 were rich in polar and hydrophobic amino acids. Several nearly invariant residues (lysine, praline, alanine, and tyrosine) at specific positions were also identified in this region (Cai et al., 2006). Moreover, HNULP1 has strong transcriptional repressive activity (Cai et al., 2006) and is a strong transcription repressor in which the DUF654 motif represents the basal transcriptional repressive activity. The repressive mechanism of DUF654 involves the recruitment of histone deacetylases. HNULP1 may act as a novel bHLH transcriptional repressor in the SRF signaling pathway. Over-expression of *hnulp1* protein in COS-7 cells inhibits the transcriptional activity of serum response factor (SRF) (Olsson et al., 2002; Cai et al., 2006).

To further study HNULP1 transcriptional regulation and the DUF654 domain transcription repression mechanism, transcription repression activity of five deletion fragments of the DUF654 domain were identified in this study. Our studies indicated that the DUF654 domain in the N-terminus had relatively stronger transcription

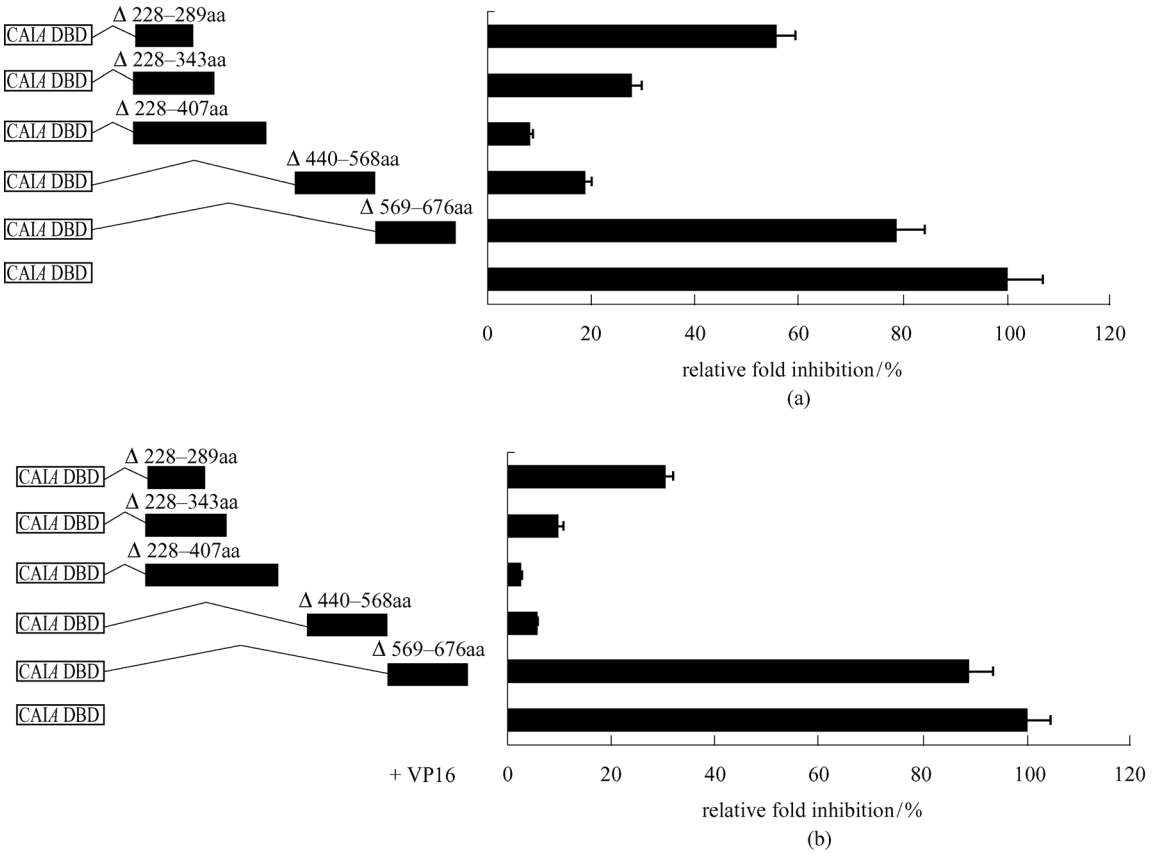

Fig. 4 Transcription inhibiting characteristics of different domains of DUF654. Note: (a) pCMV-BDΔ228–289, pCMV-BDΔ228–343, pCMV-BDΔ228–407, pCMV-BDΔ440–568, pCMV-BDΔ569–676, pCMV-BD was transiently co-transfected into COS-7 cells along with the pL8G5-Luc reporter; (b) Five deletion fragments of DUF654 domain were co-transfected with L8G5-Luc and VP-16 plasmids. The data are means, each based on three repetitions in a single transfection experiment. Each experiment was performed at least three times.

suppression activity. The more conserved the amino acids are, the stronger the transcriptional repressive activity. Bioinformatics analysis demonstrated that the DUF654 domain in the N-terminus was a highly conserved region. The sequences of DUF654 in the N-terminus were rich in polar and hydrophobic amino acids, such as lysine, praline, alanine, and tyrosine. Furthermore, the DUF654 domain in the C-terminus definitely possessed transcription repression activity. The transcription repression activity, however, was reduced along with the decreased amino acid conservation. This study therefore lays a solid foundation for research on the characteristics and functions of the DUF654 domain and the transcriptional regulation mechanism of *hnulp1*.

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