

# Solution Structure of Homeobox Transcription Factor Hex C-terminal Domain of Negatively Charged Activating Function

Shunsuke Meshitsuka<sup>1</sup>, Yuki Horie<sup>1</sup>, Kazuya Takahashi<sup>1</sup>, Ojeiru F. Ezomo<sup>1</sup>, Mohammed S. Mustak<sup>1</sup>, Takahisa Ikegami<sup>2</sup>, Tamio Noguchi<sup>3</sup>

<sup>1</sup> Institute of Regenerative Medicine and Biofunction Tottori University Graduate School of Medical Science, 68 Nishimachi Yonago Tottori 683-8503 JAPAN, <sup>2</sup>Osaka University Institute for Protein Research, Laboratory of Proteomics, Yamadaoka Suita Osaka 565-0871 JAPAN, <sup>3</sup>Osaka Ohtani University Faculty of Pharmacy, Tondabayashi Osaka 584-8540 JAPAN

**Abstract:** Transcription factor Hex (haematopoietically expressed homeobox) is a homeobox protein that regulates differentiation and development of liver and monocyte. Hex consists of three functional domains, N-terminal proline rich domain, homeodomain and acidic C terminal domain. Other than DNA binding, the function of Hex is to repress and activate transcription of target genes using multiple mechanism. However, the structure and function of Hex-C terminal domain is not yet elucidated. Hence, the present structural studies were carried out by NMR spectroscopy in order to understand the function of the Hex-C domain. We determined the NMR assignment of backbone and side chains, and obtained 3D structure of Hex-C domain in the presence of GST tag. This is the first paper to present the structure of a protein with GST tag by NMR.

**Key-Words:** transcription factor, NMR, Hex, GST, protein tag, domain structure

## 1 Introduction

Regulation of the differentiation of cells has been interested in the progress of regenerative medicine. However, the mechanism of the regulation of differentiation has not been revealed yet. Differentiation is regulated by specific transcription factors. The structures and the roles of DNA binding domains of transcription factors have been understood well [1, 2]. However, the structures and functions of the domains of transcription factors other than the DNA binding domain have scarcely been reported.

Hex (haematopoietically expressed homeobox) is one of the orphan homeobox genes, which is identified in human promyelotic leukemia cell line [3] and is found in rat [4], mouse [5], chicken [6], xenopus [7], zebrafish [8] and C.elegance [9]. It is also reported that Hex is essential for monocyte and B cell differentiation [10]. Hex is the transcription factor constituted 271 amino acids and has a proline rich repressor domain in N-terminal region (45-136 AA), a homeodomain in center (137-196 AA) and an activator domain in C-terminal region, which has many negatively charged amino acids such as glutamic acid and aspartic acid (197-271 AA) [10, 11,

12]. In early mouse embryo, Hex is expressed in anterior visceral endoderm and rostral definitive endoderm [3, 13]. In adult mouse, Hex is expressed in liver, lung, thyroid gland, spleen, vascular smooth muscle cells and multipotent haematopoietic cells [3, 14]. The knock out mouse of Hex is embryonically lethal around E10.5 due to a lack of substantial liver formation, thyroid gland formation, forebrain formation [15,16]. In general, homeobox transcription factors bind to A/T rich sequences. Hex binds directly to specific sequences such as 5'-TAAT-3', 5'-CAAG-3', 5'-ATTAA-3' using homeodomain [3,17]. Several genes such as thyroglobulin, the precursor of thyroid hormone, and gooseoid, homeodomain transcription factor involved in development of early embryo, are found to be repressed by Hex [17, 18]. In contrast, Hex activates the expression of Smemb, nonmuscle isoform of the myosin heavy chain and sodium dependent bile acid co-transporter [19]. Furthermore, the expressions of GATA-1, flk-1 and fli-1, which are the transcription factors of endothelial and blood cells for differentiation in zebrafish, are also activated by Hex [20]. In addition, it is reported that Hex binds directly to transcription factors such as

HNF-1 $\alpha$ , AP-1, GATA-2, PML and HC-8, and regulates the transcription of their target genes [21, 22, 23, 24]. However, the detail function of Hex has not been clarified yet.

The methods of the production and purification of proteins using affinity tag such as GST (glutathione S-transferase) tag, histidine tag, MBP (maltose binding protein) tag are familiar in the protein science for their easy and rapid procedure. The molecular weight of protein is restricted in NMR analyses. The protein larger than 30kDa molecular weight is not suitable for ordinary NMR analyses by the reasons of peak broadening and signal overlapping. Thus, it is thought that affinity tags except for His-tag are necessary to be removed from fusion proteins by protease. GST tag is beneficial for expression and purification in *E.coli*. However, some problems in preparing NMR samples often arise when fusion protein is cleaved by protease such as thrombin. One of the problems is nonspecific cleavage reaction by protease and another problem is decrease of the solubility of the protein after removing GST tag.

We obtained a sufficient amount of Hex C-terminal domain (Hex-C) with GST. However, Hex-C did not provide good NMR spectra for analysis on account of the aggregation of Hex, when GST tag is removed by thrombin. Therefore, we have measured NMR spectra of Hex-C without cleaving GST. Here, we present the analyses of Hex-C, which is the activator domain of Hex in view of the structural biology of the negatively charged domain of the transcription factor.

## 2 Materials and methods

### 2.1 Expression and purification of Hex-C

The cDNA of C-terminal domain of Hex (Hex-C: 206-271 amino acid residues) was subcloned into pGEX2T (GE Healthcare Bioscience, USA) BamHI cloning site. pGEX2T-Hex-C was transformed to *E.coli* BL21(DE3). BL21(DE3) pGEX2T-Hex-C was induced with a final concentration of 0.2 mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) at  $A_{600}$  nm = 0.5, and was incubated for 16 hours at 25 °C for protein production. The cells were centrifuged and suspended in lysis buffer (50 mM Tris-HCl buffer at pH 8.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 400 mM NaCl, 5 mM MgCl<sub>2</sub>, 5% (v/v) glycerol and 1 mM DTT). The cell suspension was homogenized by sonication and freeze thawing. After

the cell lysate was centrifuged, the soluble fraction was applied on a glutathione-Sepharose 4B column (GE Healthcare Bioscience, USA) equilibrated by lysis buffer and washed by PBS. GST-Hex-C was eluted from the resins by elution buffer (5 mM glutathione of reduced form in 50 mM Tris-HCl at pH 9.6). After the buffer was exchanged to 66 mM phosphate buffer (pH 7.0) containing 50 mM KCl, GST-Hex-C was concentrated by ultrafiltration of Amicon YM10 (Millipore, USA), and purified by gel chromatography of ÄKTA prime using HiLoad 16/60 Superdex 75pg column (GE Healthcare Bioscience, USA). Hex-C was obtained by hydrolysis of GST-Hex-C by thrombin (GE Healthcare Bioscience, USA) for 48h at 22 °C. Also, GST alone was synthesized by pGEX2T system separately. Hex-C and GST proteins were also purified by the gel chromatography. The typical yield GST-Hex-C was approximately 50mg of fusion protein/L of cell culture. For the production of uniformly labeled (<sup>15</sup>N and <sup>15</sup>N/<sup>13</sup>C) proteins, the expressing bacteria were grown in M9 minimal nutrient medium containing 1.5 g <sup>15</sup>NH<sub>4</sub>Cl or 1.5 g <sup>15</sup>NH<sub>4</sub>Cl / 2.0 g [U-<sup>13</sup>C]-glucose. All the procedures except for incubation and protease reaction were performed at 4 °C.

### 2.2 NMR spectra and backbone assignment of Hex-C

NMR measurements were performed by Varian Inova (500 MHz) spectrometer equipped with a triple resonance probe and z-axis pulsed-field gradient. Chemical shift of <sup>1</sup>H were referenced to water at 4.75 ppm (at 25 °C), whereas the  $\gamma^{13}\text{C}/\gamma^1\text{H}$  and  $\gamma^{15}\text{N}/\gamma^1\text{H}$  ratios were used for indirect referencing of the <sup>13</sup>C and <sup>15</sup>N chemical shifts. Backbone resonances were assigned by a combination of <sup>15</sup>N-HSQC (<sup>15</sup>N-Heteronuclear Single Quantum Coherence), CBCANH, CBCACONH, HNCO and HNCACO. The  $\alpha\text{H}$  and  $\beta\text{H}$  resonances and the side chain protons were assigned using TOCSY-<sup>15</sup>N-HSQC, HCCONH, and <sup>13</sup>C-HSQC spectra. The aromatic side chain proton and carbon atoms were identified by analyses of DQF-COSY, HCCH-TOCSY, NOESY spectra. NOE cross-peaks were detected using two dimensional H-H NOESY, and three dimensional NOESY-<sup>15</sup>N-HSQC and NOESY-<sup>13</sup>C-HSQC spectra. NOE mixing time was 150 ms for spectra used to obtain distance constraints. The measurement condition of <sup>15</sup>N-HSQC were ni=512, nt=32, in 66 mM phosphate buffer (pH7.0), 50mM KCl, 1 mM to 5 mM DTT and 10% D<sub>2</sub>O. The condition of 3D

measurement were at  $128(t_1) \times 96(t_2) \times 1024(t_3)$  of 16 transients in 66 mM phosphate buffer (pH7.0), 50mM KCl, 1 mM to 5 mM DTT and 10% D<sub>2</sub>O. The temperature of all NMR experiments were carried out at 25 °C. All the NMR spectra were processed using NMR-Pipe [25] and Sparky software [26].

### 2.3 Distance, dihedral angle, hydrogen-bond restraints, and structure calculations

The backbone dihedral angles ( $\phi$  and  $\psi$ ) were calculated by analyzing the  $^{13}\text{C}_\alpha$  and  $^{13}\text{C}_\beta$ ,  $^{13}\text{C}'$  and  $^{15}\text{N}$  chemical shifts with the TALOS program that predicts the backbone torsion angles from the amino acid sequence and chemical-shift information [27]. During later stages of structure calculations, the hydrogen bonds were added to the  $\alpha$  helices as additional restraints. The hydrogen-bonding restraints were defined as 1.83-2.3 Å for the H-O distance and 2.8-3.3 Å for the N-O distance. Distance restraints were derived from 3D  $^{15}\text{N}$ -edited NOESY (150 ms mixing time) and 3D  $^{13}\text{C}$ -edited NOESY (150 ms mixing time) experiments. Using Sparky, the NOESY cross-peak volumes/intensities were obtained and converted into distance restraints. Structure calculations were performed by CYANA [28]. The surface charges were calculated by Discovery Studio.

### 2.4 Chemicals

$^{15}\text{NH}_4\text{Cl}$  and  $[\text{U-}^{13}\text{C}]$ -glucose were purchased from Isotech Inc., USA. Deuterium oxide was obtained from CEA, France.

## 3 Results

The signal peaks were resolved well in the  $^{15}\text{N}$ -HSQC spectrum of Hex-C in the presence of GST tag. On the other hand, the signals of Hex-C after removal of GST tag in the  $^{15}\text{N}$ -HSQC spectrum were not resolved well, presumably due to the aggregation of the protein. In addition  $^{15}\text{N}$ -HSQC spectrum of GST alone was also measured in the same condition as GST-Hex-C. The signals from GST were very weak and completely different from GST-Hex-C. Therefore, the signals observed in the spectrum of  $^{15}\text{N}$ -HSQC of GST-Hex-C were assumed only from Hex-C even in the presence of GST-tag.

The uniformly  $^{15}\text{N}$ - and  $^{13}\text{C}$ -labeled Hex-C was used to obtain the main- and side-chain assignments of the  $^1\text{H}$ ,  $^{15}\text{N}$ , and  $^{13}\text{C}$  resonances using the standard set of triple-resonance 3D experiments. The backbone

signals of 66 amino acid residues of Hex-C and 6 amino acid residues of linker connecting GST and Hex-C were assigned by the sequential connection of CBCANH and CBCACONH spectra. The results were in good agreement with the assignment from the connection of HNCO and HNCACO spectra. Consequently, all the peaks in  $^{15}\text{N}$ -HSQC spectrum of Hex-C were assigned. Further, we obtained the chemical shifts of the peaks from the backbone and other atoms such as  $\text{C}_\alpha$ ,  $\text{C}_\beta$  and amide N and H of Hex-C. The chemical shifts of sidechain atoms of amino acids were also determined well by 3D measurement. In addition, there are five proline residues in the Hex-C domain, and they were expected to be in trans configuration based on  $\text{H}\alpha\text{-H}\delta/\text{H}\delta'$  NOE data.

An initial assessment of the secondary structure of Hex-C domain was made using the deviations of assigned chemical shifts from random coil. The chemical shift index calculated from chemical shifts of  $\text{C}_\alpha$  of Hex-C and those of random coil expected that Hex-C has  $\alpha$ -helices at Asp207 to Asp221, Ser224 to Gln236 and Ser252 to Gly266.

The nuclei belong to the linker peptide moiety connecting Hex-C domain and GST tag exhibited strong peaks in  $^{15}\text{N}$ -HSQC, but did not show any NOE peaks. Therefore, the linker peptide is supposed to be completely flexible. This is the reason why the signals from Hex-C were independently observed even in the presence of GST tag.

The NOE peaks in the NOESY- $^{15}\text{N}$ -HSQC spectra were analyzed, and  $\alpha$ -helices were expected from the sequential observation of NOE peaks between  $\text{H}_\alpha(i)$  and  $\text{NH}(i+3)$ , indicating that Hex-C has helices from D210 to L222 and from E248 to E262.

All NOE cross-peaks in the NOESY- $^{15}\text{N}$ -HSQC and NOESY- $^{13}\text{C}$ -HSQC spectra were used by CYANA. The final set of structure calculations were performed using a total restraints of intraresidue, sequential, short range, medium range, and long range. In addition, dihedral angle ( $\phi$  and  $\psi$ ) and hydrogen-bond restraints were used in the structure calculations. The residues in the linker portion between GST tag and Hex-C domain have no NOE signals, so the residues in the linker portion were excluded from the structure calculation.

From the present calculation the 20 lowest energy structures are superimposed. These structures exhibit no distance and dihedral restraint violations greater than 0.5 Å and 5 degree, respectively. The three

dimensional structure of Hex-C domain has four  $\alpha$ -helices (helix 1: E208-S215, helix 2: L222-K228, helix 3: S231-Q236, helix 4: S249-E258). Noteworthy is the presence of a hydrophobic core which is formed by the residues of L209, L212, P239, A242 and F268 to stabilize the domain structure. In addition, there is no homologous domains but weak resemblance is seen in two proteins such as reverse gyrase (1GKU-B) and fumarate reductase (1KF6-D) in the DALI database [29].

The aromatic ring protons of Y267 and F268 were observed clearly by DQCOSY but could not be discriminated in  $\epsilon_1$  and  $\epsilon_2$ , and also in  $\delta_1$  and  $\delta_2$  positions. Therefore, the aromatic ring is flipping in the NMR time-scale. However, in the oxidized state two protons were separately observed by fixing the aromatic rings. The oxidized form was reduced reversibly to the native form by the addition of DTT. The location of C217 and C235 are very close to each other in the calculated structure.

## 4 Discussion

This is the first paper to present the analysis of NMR spectra of a protein with GST tag. We have assigned the  $^1\text{H}$ ,  $^{15}\text{N}$  and  $^{13}\text{C}$  signals of the backbone and the side chains of Hex-C. It is thought that the protein larger than 30kDa is not suitable for NMR analyses on account of peak broadening and signal overlapping. GST is 27 kDa protein which consists of seven  $\alpha$ -helices and five  $\beta$ -sheets, those compose the tight structure. In addition, GST forms homodimer, of which apparent molecular weight is 54 kDa. Therefore, the almost all signals of GST in NMR spectra were broadened and overlapped. On the other hand, it seems likely that the mobility of the Hex-C domain (6 kDa) in the fusion protein is sufficiently well compared with the GST domain. The motion of the linker peptide is so random and free that NOE signals of the linker part may not be observed at all. In consequence, the signals of Hex-C appeared, and those of GST were too broad to be resolved and too weak to be discriminated in the spectrum of the GST fusion protein. We thought that the high solubility and high stability of GST fusion proteins are beneficial for preparation and the use of affinity tag is convenient for separation. Also in our preparation we often encounter the precipitation of a target protein after cleavage of GST tag from the fusion protein.

GST-fused proteins and peptides have been analyzed by X-ray crystallography [30]. The structures of GST moiety are identical, and also the final structures of the fused segments are not affected by GST fusion.

The structures of the DNA binding domains in transcription factors have been studied and have been analyzed well [1, 2]. However, the structures of activator or repressor domain of transcription factor have not sufficiently revealed yet. Therefore, we tried to carry out the NMR analyses of Hex-C fused with GST and assigned the signals from backbone and side chain atoms to determine the 3D structure. Hex-C domain consists of 19 acidic amino acid residues. These residues are expected to be exposed to the bulk solvent in the structure forming a contiguous surface on the Hex-C domain that may correspond to the protein-protein interaction in the activation of transcription of other transcription factors such as GATA-2 and HNF-1 $\alpha$ . Calculated surface charge of Hex-C domain is predominantly negative. The functional significance of this surface-charge distribution is unclear. Because the surface of the double strand DNA is negatively charged on account of the phosphate groups. Hex-C domain is repulsive to DNA, to which the neighbor domain of homeodomain binds.

Hex-C in the presence of GST is expected to have helices and no sheet from the data of chemical shift indices and it is shown by the structure calculation. In general, it is thought that a domain, which has abundant acidic amino acid residues, tends to form a random coil on account of the electrostatic repulsion. However, transcription factors such as VP16, GAL4 and TFE3 have helices in the acidic amino acid residues rich domain [31]. These transcription factors interact with various proteins and regulate transcription. For instance, RAP74 is one of the proteins which bind to the largest subunit of RNA polymerase II. RAP74 has  $\alpha$ -helix consisting of acidic rich residues. RAP74 binds directly to the positively charged pockets of large subunit of RNA polymerase II using this  $\alpha$ -helix [32, 33]. It is expected that Hex-C may interact with some proteins using negatively charged helices and functions as activator. Here, we have determined the three dimensional structure of Hex-C and looked for the homological similarity of the structure with other proteins to explain the functions of Hex as activator in detail. However, we have not found the suitable homological structures so far.

In summary, the NMR signals of free Hex-C

alone were not resolved well in  $^{15}\text{N}$ -HSQC. To the contrary, Hex-C were resolved well in the NMR spectra of GST-Hex-C. In this study we assigned the backbone and side chain signals of 66 amino acid residues of Hex-C and showed that Hex-C consists of four helices. The present results revealed that NMR structural analysis of a protein is possible in the presence of GST tag. The success of assignment of small proteins with GST tag by  $^{13}\text{C}$ ,  $^{15}\text{N}$  labeling is beneficial for analyses and characterization of the proteins of insufficient solubility, or lesser stability without GST tag.

#### References:

- [1] Soufi A, Jayaraman PS, PRH/Hex: an oligomeric transcription factor and multifunctional regulator of cell fate. *Biochem J*. Vol.412, No.3, 2008, pp.399-413.
- [2] Gehring WJ, Klotter U, Suga H. Evolution of the Hox gene complex from an evolutionary ground state. *Curr Top Dev Biol*. Vol.88, No.1, 2009, pp.35-61.
- [3] Crompton MR, Bartlett TJ, MacGregor AD, Manfioletti G, Buratti E, Giancotti V, Goodwin GH. Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. *Nucleic Acids Res*. Vol.20, No.21, 1992, 5661-7.
- [4] Tanaka T, Inazu T, Yamada K, Myint Z, Keng VW, Inoue Y, Taniguchi N, Noguchi T. cDNA cloning and expression of rat homeobox gene, Hex, and functional characterization of the protein. *Biochem J*. Vol.339, No.1, 1999, pp.111-7.
- [5] Keng VW, Fujimori KE, Myint Z, Tamamaki N, Nojyo Y, Noguchi T. Expression of Hex mRNA in early murine postimplantation embryo development. *FEBS Lett*. Vol.426, No.2, 1998, pp.183-6.
- [6] Yatskievych TA, Pascoe S, Antin PB. Expression of the homeobox gene Hex during early stages of chick embryo development. *Mech Dev*. Vol.80, No.1, 1999, pp.107-9.
- [7] Newman CS, Chia F, Krieg PA. The XHex homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech Dev*. Vol.66, No.1-2, 1997, pp.83-93.
- [8] Ho CY, Houart C, Wilson SW, Stainier DY. A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr Biol*. Vol. 9, No.19, 1999, pp.1131-4.
- [9] Morck C, Rauthan M, Wagberg F, Pilon M. pha-2 encodes the *C. elegans* ortholog of the homeodomain protein HEX and is required for the formation of the pharyngeal isthmus. *Dev Biol*. Vol. 272, No.2, 2004, pp.403-18.
- [10] Keng VW, Yagi H, Ikawa M, Nagano T, Myint Z, Yamada K, Tanaka T, Sato A, Muramatsu I, Okabe M, Sato M, Noguchi T. Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem Biophys Res Commun*. Vol.276, No.3, 2000, pp.1155-61.
- [11] Kikkawa E, Hinata M, Keng VW, Myint Z, Sato A, Yamada K, Tanaka T, Noguchi T. Sp family members stimulate transcription of the hex gene via interactions with GC boxes. *J Biochem (Tokyo)*. Vol. 130, No.6, 2001, pp.885-91.
- [12] Sato A, Keng VW, Yamamoto T, Kasamatsu S, Ban T, Tanaka H, Satoh S, Yamada K, Noguchi T. Identification and characterization of the hematopoietic cell-specific enhancer-like element of the mouse hex gene. *J Biochem (Tokyo)*. Vol. 135, No. 2, 2004, pp. 259-68.
- [13] Jones CM, Broadbent J, Thomas PQ, Smith JC, Beddington RS. An anterior signalling centre in *Xenopus* revealed by the homeobox gene XHex. *Curr Biol*. Vol. 9, No. 17, 1999, pp. 946-54.
- [14] Thomas PQ, Brown A, Beddington RS. Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development*. Vol. 125, No. 1, 1998, pp. 85-94.
- [15] Martinez-Barbera JP, Beddington RS. Getting your head around Hex and Hexx1: forebrain formation in mouse. *Int J Dev Biol*. Vol. 45, No.1, 2001, pp. 327-36.
- [16] Bogue CW, Zhang PX, McGrath J, Jacobs HC, Fuleihan RL. Impaired B cell development and function in mice with a targeted disruption of the homeobox gene Hex. *Proc Natl Acad Sci U S A*. Vol. 100, No.2, 2003, pp. 556-61.
- [17] Pellizzari L, D'Elia A, Rustighi A, Manfioletti G, Tell G, Damante G. Expression and function of the homeodomain-containing protein Hex in thyroid cells. *Nucleic Acids Res*. Vol. 28, No.13, 2000, pp. 2503-11.
- [18] Brickman JM, Jones CM, Clements M, Smith JC, Beddington RS. Hex is a transcriptional repressor that contributes to anterior identity and suppresses Spemann organiser function. *Development*. Vol. 127, No.11, 2000, pp. 2303-15.
- [19] Kasamatsu S, Sato A, Yamamoto T, Keng VW, Yoshida H, Yamazaki Y, Shimoda M, Miyazaki J, Noguchi T. Identification of the transactivating

- region of the homeodomain protein, hex. *J Biochem (Tokyo)*. Vol. 135, No.2, 2004, pp. 217-23.
- [20] Liao W, Ho CY, Yan YL, Postlethwait J, Stainier DY. Hhex and Scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development*. Vol. 127, No. 20, 2000, pp 4303-13.
- [21] Schaefer LK, Wang S, Schaefer TS. Functional interaction of Jun and homeodomain proteins. *J Biol Chem*. Vol. 276, No.46, 2001, pp. 43074-82.
- [22] Minami T, Murakami T, Horiuchi K, Miura M, Noguchi T, Miyazaki J, Hamakubo T, Aird WC, Kodama T. Interaction between hex and GATA transcription factors in vascular endothelial cells inhibits flk-1/KDR-mediated vascular endothelial growth factor signaling. *J Biol Chem*. Vol. 279, No.20, 2004, pp. 20626-35.
- [23] Topcu Z, Mack DL, Hromas RA, Borden KL. The promyelocytic leukemia protein PML interacts with the proline-rich homeodomain protein PRH: a RING may link hematopoiesis and growth control. *Oncogene*. Vol. 18, No.50, 1999, pp. 7091-100.
- [24] Bess KL, Swingler TE, Rivett AJ, Gaston K, Jayaraman PS. The transcriptional repressor protein PRH interacts with the proteasome. *Biochem J*. Vol. 374, 2003, pp.667-75.
- [25] Comilescu, G., Delaglio, F., and Bax, A. Protein backbone angle restraints from searching a database for chemical shift and sequence homology, *J Biomol NMR* Vol.13, No.3, 1999, pp.289-302.
- [26] Goddard TD, Kneller DG, SPARKY 3, University of California, San Francisco.
- [27] Delaglio, F., Grzesiek, S., Vuister, G., Zhu, G., Pfeifer, J., and Bax, A. (1995) *J. Biomol. NMR*. Vol. 6, No.3, 1995, pp.277-293
- [28] Herrmann, T., Güntert, P., and Wu'thrich, K. *J. Biomol. NMR*, Vol. 24, 2002, pp.171-189
- [29] Holm L, Kaariainen S, Rosenstrom P, Schenkel A. Searching protein structure databases with DaliLite v.3. *Bioinformatics*, Vol.24, No.23, 2008, pp.2780-2781.
- [30] Zhan Y, Song X, Zhou GW. Structural analysis of regulatory protein domains using GST-fusion proteins, *GENE*, Vol.281, 2001, pp.1-9.
- [31] Seipel K, Georgiev O, Schaffner W. Different activation domain stimulate transcription from remote ('enhancer') and proximal ('promoter') positions. *EMBO J*. Vol. 11, No.13, 1992, pp.4961-8.
- [32] Funk JD, Nedialkov YA, Xu D, Burton ZF. A key role for the alpha 1 helix of human RAP74 in the initiation and elongation of RNA chains. *J Biol Chem*. Vol. 277, No.49, 2002, pp.46998-7003.
- [33] Nguyen BD, Abbott KL, Potempa K, Kobor MS, Archambault J, Greenblatt J, Legault P, Omichinski JG. NMR structure of a complex containing the TFIIF subunit RAP74 and the RNA polymerase II carboxyl-terminal domain phosphatase FCP1. *Proc Natl Acad Sci USA*. Vol.100, No.10, 2003, pp.5688-93.