The Existence of a Putative Regulatory Element in 3'-Untranslated Region of Proto-oncogene HOX11’s mRNA

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HOXII encodes a homeodomain-containing transcription factor which directs the development of the spleen during embryogenesis. While HOXII expression is normally silenced through an unknown mechanism in all tissues by adulthood, the deregulation of HOXII expression is associated with leukemia, such as T-cell acute lymphoblastic leukemia. The elucidation of regulatory elements contributing to the molecular mechanism underlying the regulation of HOXII gene expression is of great importance. Previous reports of HOXII regulatory elements mainly focused on the 5'-flanking region of HOXII on the chromosome related to transcriptional control. To expand the search of putative cis-elements involved in HOXII regulation at the post-transcriptional level, we analyzed HOXII mRNA 3'-untranslated region (3'UTR) and found an AU-rich region. To characterize this AU-rich region, in vitro analysis of HOXII mRNA 3'UTR was performed with human RNA-binding protein HuR, which interacts with AU-rich element (ARE) existing in the 3'UTR of many growth factors' and cytokines' mRNAs. Our results showed that the HOXII mRNA 3'UTR can specifically bind with human HuR protein in vitro. This specific binding could be competed effectively by typical ARE containing RNA. After the deletion of the AU-rich region present in the HOXII mRNA 3'UTR, the interaction of HOXII mRNA 3'UTR with HuR protein was abolished. These findings suggest that HOXII mRNA 3'UTR contains cis-acting element which shares similarity in the action pattern with ARE-HuR interactions and may involve in the post-transcriptional regulation of the HOXII gene.

Keywords: AU-rich element, HOXII, Proto-oncogene, T-cell acute lymphoblastic leukemia, 3'-untranslated region

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are targeted for post-transcriptional regulation by virtue of cis-elements in the 3' untranslated region (3'UTR) (Shaw and Kamen, 1986; Sachs, 1993; Levy et al., 1996). So is there any cis-element in the 3' UTR of HOX11 mRNA? A scan of putative cis-element in HOX11 mRNA sequence was performed, and we found a segment of AU-rich sequence in 3'UTR of HOX11 mRNA, which is similar to the AU-rich element (ARE). We anticipate this AU-rich segment in 3'UTR of HOX11 may be a cis-element. In order to characterize this element, RNA-protein interaction analysis is performed using human RNA binding protein HuR, which can bind specifically with typical cis-element ARE (Ma et al., 1996). In vivo studies have shown HuR can specifically bind and stabilize ARE-containing mRNA (Fan and Steitz, 1998; Peng et al., 1998; Levy et al., 1998). Over-expression of HuR is found in tumors of central nervous system, and strong HuR expression was limited to high grade malignancies (Nabors et al., 2001). With RNA-protein interaction analysis, we can evaluate the binding affinity and specificity of HOX11 mRNA 3'UTR with HuR protein, compare the binding features in the competition assay with typical ARE containing RNA, and determine the main binding site by deletion analysis.

Materials and Methods

Cloning and construction of human HOX11 mRNA 3'UTR in vitro transcription plasmids The human HOX11 mRNA 3'UTR was cloned by PCR as follows. The genomic DNA was extracted from human blood cells and amplified using oligodeoxynucleotides described as forward, 5'-GGTCCAGAAAGCCAGGGAGGT3'; reverse, 5'-CACCCGACAGGGGCGAC-3'. The PCR products were subcloned into a pUCm-T vector and transformed with E. coli BL21, transformed with pGEX-HuR, was diluted in 50 ml LB medium at a proportion of 0.5 : 50 and incubated at 37°C for 20 h. The culture was induced with IPTG (0.04 mM). After 4 h of further growth at 30°C E. coli cells were spun down and resuspended in 5 ml of buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 1 mM ethylenediamine tetracetic acid (EDTA)). The cells were lysed by adding lysozyme to a final concentration of 0.2 mg/ml and Triton X-100 to 3%, respectively. The lysate was centrifuged at 12,000 × g for 30 min. The supernatant was mixed with 300 µl of 50% slurry of Glutathione Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) and incubated with gentle agitation at room temperature for 1 h. Mixtures were centrifuged at 1000 × g for 5 min. After washing the pellet three times with 1.5 ml buffer A, the bound protein was eluted with elution buffer (50 mM Tris pH 8.0, 10 mM reduced glutathione). The GST-HuR fusion protein concentration was measured using Bradford assay (Bradford, 1976).

Preparation of RNA transcripts Plasmid DNAs were digested with the appropriate restriction enzymes and transcribed using MAXIscript in vitro transcription kit (Ambion). For the synthesis of biotin-labeled RNA, 30% CTP was replaced by biotin-14-CTP (Invitrogen, San Diego, USA); for the synthesis of unlabeled RNA, no biotin-14-CTP was added. pUCm-HOX11 was linearized with Sal I and transcribed with T7 RNA polymerase, yielding an 888 nucleotides transcript (called HOX11-3'UTRA) containing the sequence from residues 1003 to 1850 of HOX11 mRNA (GenBank accession no. M75952), which located in downstream of the HOX11 mRNA stop codon, pUCm-HOX11∆ was linearized with Sal I and transcribed with T7 RNA polymerase, yielding an 888 nucleotides transcript (called HOX11-3'UTRA) containing the sequence from residues 1003 to 1850 of HOX11 mRNA (GenBank accession no. M75952), which located in downstream of the HOX11 mRNA stop codon while lacking the AU-rich sequence from residues 1241 to 1290 of HOX11 mRNA. pUCm-HOX11 was linearized with Hind III, yielding a transcript of 214 nucleotides containing the sequence from residues 568 to 781 downstream of the c-fos mRNA stop codon (Shyu et al., 1991). pSP6 Shy (human γ-globin) was linearized with Sma I, yielding 165 nucleotides, containing 80 nucleotides of coding sequence and 85 nucleotides of 3'-untranslated regions (Chung et al., 1996). The biotin-labeled RNA concentration was determined by measuring OD_{260} with Gene Spec III (Naka instruments Co. Ltd.)

Purification of GST-HuR fusion protein The GST (glutathione S-transferase)-HuR fusion protein was expressed from pGEX-HuR (Ma et al., 1996). An overnight culture of E. coli BL 21, transformed with pGEX-HuR, was diluted in 50 ml LB medium at a proportion of 0.5 : 50 and incubated at 37°C for 2 h. The culture was induced with IPTG (0.04 mM). After 4 h of further growth at 30°C E. coli cells were spun down and resuspended in 5 ml of lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM ethylenediamine tetracetic acid (EDTA)). The cells were lysed by adding lysozyme to a final concentration of 0.2 mg/ml and Triton X-100 to 3%, respectively. The lysate was centrifuged at 12,000 × g for 30 min. The supernatant was mixed with 300 µl of 50% slurry of Glutathione Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) and incubated with gentle agitation at room temperature for 1 h. Mixtures were centrifuged at 1000 × g for 5 min. After washing the pellet three times with 1.5 ml buffer A, the bound protein was eluted with elution buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1 mM reduced glutathione). The GST-HuR fusion protein concentration was measured using Bradford assay (Bradford, 1976).

Electrophoretic mobility shift assay Excess amounts of RNA and bovine serum albumin (BSA) were used as non-specific competitor to assure the specificity of mRNA-protein interaction in electrophoretic mobility shift assay (EMSA). Reaction mixtures (0.02 ml) contained 30 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml RNA, 0.25 mg/ml BSA, 40 fmol biotin-labeled RNA transcripts or 200 fmol biotin-labeled γ-globin and protein as indicated. Mixtures were incubated at 37°C for 10 min. Following incubation, 2 µl of a dye mixture (50% glycerol, 0.025% bromphenol blue, 0.025% xylene cyanol) was added and the reaction mixture was immediately loaded on a 1% agarose gel in TBE buffer (40 mM Tris borate, 1 mM EDTA, pH 8.0). The gel was then photographed at 70 V for 45 min.

Detection of the biotin-labeled RNA and quantification of its optical density The RNA was transferred from gel to Zeta-probe nylon membrane (BIO-RAD Co., Richmond, USA) using standard capillary transferring (Sambrook et al., 1992). The RNA was cross-linked to membrane by exposure to 302 nm ultraviolet radiation on a UV transilluminator for 3 min. The membrane was incubated in 10 ml block solution (0.1 M Tris, pH 7.5, 0.1 M NaCl, 2 mM MgCl$_2$, 3% bovine serum albumin in Fraction V) at 30°C for 1 h and then incubated at 30°C with 0.85 µg/ml Streptavidin Alkaline

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Phosphatase (Promega, Madison, USA) in 10 ml AP 7.5 buffer (0.1 M Tris, PH 7.5, 0.1 M NaCl, 2 mM MgCl₂) for 10 min. After being washed twice with 100 ml AP 7.5 buffer for 10 min and once with 100 ml AP 9.5 buffer (0.1 M Tris, PH 9.5, 0.1 M NaCl, 50 mM MgCl₂) for 10 min, the membrane was incubated with 2.5 mg nitroblue tetrazolium (NBT) and 1.25 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 7.5 ml AP 9.5 buffer at room temperature for 15 min, and the TE buffer (10 mM Tris PH 8.0, 1 mM EDTA) was added to stop the reaction.

After the color development reaction of biotin-labeled RNA, the nylon membrane was scanned by a scanner. The optical density of each band was quantified using ImageQuant version 5.2 software (Molecular Dynamics). The integrated density of all the pixels in the area of each band was quantified and adjusted by a subtraction of density in nearest blank area of the same size (background). This value is the optical density of the band. A series quantities of biotin-labeled HOX1-3'UTR samples were processed and the optical densities of the corresponding band were measured. In the range of the processed RNA quantities, the optical density of biotin-labeled HOX1-3'UTR versus the logarithm value of RNA quantities reveals a straight line. With this standard curve, the biotin-labeled HOX1-3'UTR quantities could be calculated according to the optical density of biotin-labeled HOX1-3'UTR on the membrane within the linear range and the results of EMSA were quantitatively analyzed (Li et al., 2004).

The apparent equilibrium dissociation constant $K_d$ which represents the binding affinity between two molecules, is calculated based on a plotting of $\log_{10} \left\{ [\text{RNA-Protein}] / [\text{RNA}] \right\}$ on the Y-axis and $\log_{10} [\text{protein}]$ on the X-axis (Chung et al., 1996).

### Results

The HOX11-3'UTR can be bound with HuR protein in a concentration-dependent pattern. In electrophoretic mobility shift assay (EMSA), purified GST-HuR fusion protein was added to the RNA-binding buffer at indicated amounts immediately prior to the binding reaction. As shown in Fig. 1, purified GST-HuR fusion protein converts the HOX11-3'UTR to a stable protein-RNA complex that migrates slowly on agarose gel electrophoresis (Fig. 1, lanes 3-8) than the HOX11-3'UTR alone (Fig. 1, lane 1). When the concentration of GST-HuR increased from 25 nM to 800 nM, the amount of the formed protein-RNA complex increased, and the unbound HOX11-3'UTR reduced gradually (Fig. 1, lanes 3-8). The complex is formed between HOX11-3'UTR and HuR protein, since no complex was observed by HOX11-3'UTR and purified GST (Fig. 1, lane 2). When the concentration of GST-HuR was at 400 nM to 800 nM, retarded HOX11-3'UTR and HuR complex varied in size and distributed in certain range (Fig. 1, lanes 7 and 8). The result indicated that the HOX11-3'UTR can be bound by human HuR protein specifically in a concentration-dependent pattern.

Quantitative analysis to the specific binding of the HOX11-3'UTR with HuR protein A series of biotin-labeled HOX11-3'UTR samples whose quantities are 1.27, 3.81, 11.4, 34.3 and 103 fmol respectively were processed and their corresponding optical densities were measured after electrophoresis and being transferred to the nylon membrane. A plot of the optical density of biotin-labeled HOX11-3'UTR versus the logarithm value of HOX11-3'UTR quantities revealed a straight line within this range and served as the standard curve. With this standard curve of the relationship between the optical densities of biotin-labeled HOX11-3'UTR and the logarithm value of quantities of the corresponding RNA, the biotin-labeled RNA quantity on the membrane was determined after calculation.

This quantitative approach was employed to measure RNA quantities in lanes 3-8 of Fig. 1. The integrated density of all the pixels in the area of each unbound HOX11-3'UTR and retarded HOX11-3'UTR and HuR complex was quantified and adjusted by a subtraction of density in nearest blank area of the same size (background), respectively. Based on the value of the optical densities and the standard curve, HOX11-3'UTR quantities were determined and the values of complex/free RNA were obtained. A plot of the logarithm value of complex/free RNA versus the logarithm value of HuR concentration reveals a straight line with an intersect on the X axis at 120 nM (Fig. 2), which reveals the binding of HuR with HOX11-3'UTR is a simple molecular reaction with an apparent $K_d$ of 120 nM.

The binding of HOX11-3'UTR with HuR protein can be competed by typical ARE-containing RNA We next determined whether this specific interaction has similarity with the typical ARE-protein interaction. RNA transcript of full length AU-rich sequence in the c-fos 3'UTR (AUF1) which contains ARE of the c-fos mRNA, and RNA transcript of human $\gamma$-globin 3' sequence ($\gamma$-globin) which does not contain any ARE, were used as the competitors in the competition experiment. The competition experiment was made to check whether HOX11-3'UTR would be displaced by AUF transscripts for binding with HuR protein. As shown in Fig. 3, GST-HuR protein did not bind to $\gamma$-globin RNA, which
A Putative Regulatory Element in HOX11's mRNA was used as a negative control (Fig. 3, lanes 1 and 2). Unlabeled AUFL transcripts, acted as competitor, were added to the RNA-binding buffer at about 20 times excess amounts of biotin-labeled HOX11-3'UTR transcripts prior to the binding reaction. The binding of HuR to the HOX11-3'UTR transcripts was greatly reduced (Fig. 3, lane 5) comparing with the retarded HuR- HOX11-3'UTR complex (Fig. 3, lane 4). No reduced reaction was observed with unlabeled γ-globin and AUFL transcripts acted as competitor at about 20 times excess amounts of biotin-labeled HOX11-transcripts.

An AU-rich region in HOX11-3'UTR contributes to its specific binding with HuR Analysis of the sequence revealed that the HOX11 mRNA 3'UTR contains an AU-rich region of 50 nucleotides in the proximal portion of the 3'UTR. The location of this AU-rich sequence is from nt 1241 to 1290 of HOX11 mRNA (GenBank accession no. M75952). Since HOX11 mRNA 3'UTR and ARE have similarity in the binding site or action pattern with HuR protein, this AU-rich region may participate in the specific binding of HOX11 mRNA 3'UTR with HuR protein. To verify this hypothesis, a new plasmid pUCm-HOX11∆ was constructed to produce transcript (designated HOX11-3'UTR∆) that contains HOX11 mRNA 3'UTR but lacks the AU-rich sequence (Fig. 4A).

We first compared the binding ability of HOX11-3'UTR and HOX11-3'UTR∆ with HuR protein. As shown in Fig. 4B, when the concentration of HuR is 200 nM, the HOX11-3'UTR∆ and HuR formed little complex, compared with a totally obvious complex formed by HOX11-3'UTR and HuR. Subsequent binding analysis of HOX11-3'UTR and HOX11-3'UTR∆ with increased concentration of HuR was made and the results were quantitatively assayed. Fig. 5 shows the difference of binding ability between HOX11-3'UTR with HuR and HOX11-3'UTR∆ with HuR. When the HuR concentration is 800 nM, the percentage RNA bound value of HOX11-3'UTR∆ is less than 15%. Compared with the strong
Discussion

In this paper, our results demonstrated that the 3'UTR of HOX11 mRNA could be bound by HuR protein specifically and this binding could be specifically competed by AUFL. An AU-rich segment contributes greatly to this specific binding. The results suggest that 3'UTR of HOX11 mRNA contains a cis-element that is most likely to play a role in the post-transcriptional regulation.

Previous reports mainly focused on HOX11 regulation at the transcriptional level. Both positive and negative elements in the promoter of HOX11 5' non-coding region on the chromosome were found (Brake et al., 1998; Brake et al., 2002). A recent research found that expression of HOX11 in T-ALL is associated with extensive demethylation of the proximal HOX11 promoter (Watt et al., 2000). It was reported that HOX11 expression is dependent on protein synthesis and its up-regulation in T cells requires a tyrosine phosphorylation signal (Zhang et al., 1995). In addition to the transcriptional regulatory elements, post-transcriptional regulatory elements are emerging as an important control element for gene expression in eukaryotes (Sachs 1993; Ross 1995). ARE is the best-studied cis-element in mammalian mRNA, which will influence the mRNAs stability by acting with RNA-binding proteins. Many growth factors and cytokines integral to tumor proliferation and angiogenesis have ARE within the 3'UTR that govern transcript half-life (Chen and Shyu, 1995). HOX11 mRNA 3'UTR contains a cis-element suggests there may exist a post-transcriptional regulation of the HOX11 gene expression. This post-transcriptional regulation may be a complementary mechanism for the development of spleen during embryogenesis or the deregulation of HOX11 in T-ALL, which is believed to be a key event in the development of leukemia (Lu et al., 1992).

Fig. 5. Comparison of the binding ability between HuR with HOX11-3'UTR and HuR with HOX11-3'UTRΔ. EMSA of HuR protein with biotin-labeled HOX11-3'UTR or HOX11-3'UTRΔ was performed as described above. The results were quantitatively analyzed and plotted.

The molecular mechanism of post transcriptional regulation depends on the interaction between the cis-element on the HOX11 mRNA 3'UTR and its binding factors. Many factors have been found to selectively bind the AU- and U-rich sequence, while only two of these proteins, HuR (Ma et al., 1996; Fan and Steitz, 1998; Brennan and Steitz 2001) and hnRNP D (AUF1) (Zhang, W. et al., 1993; Sarkar et al., 2003; Bremer et al., 2003) have been demonstrated to alter the stability of ARE-containing mRNA in vivo. HuR is a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of human RNA-binding proteins (Ma et al., 1996). Overexpression of HuR protein stabilizes ARE-containing mRNA in vivo, which influences the final protein expression of the ARE-containing mRNA (Fan and Steitz, 1998; Peng et al., 1998; Levy et al., 1998). Recent evidences have suggested that HuR-ARE specific interaction plays a role in carcinogenesis by stabilizing ARE-containing mRNAs of growth factors and cytokines integral to tumor proliferation and angiogenesis. For example, HuR was found over-expressed in tumors of central nervous system, strong HuR expression was limited to high grade malignancies, and it was also found HuR could bind specifically to the ARE of angiogenic factors including vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2) and interleukin-8 (IL-8) (Nabors et al., 2001). In human colon cancer, the binding of HuR with the 3'UTR of cyclin-dependent kinase inhibitor p21 and carcinogenesis related gene VEGF, COX-2, IL-8 improves mRNA’s stability (Wang, 2000; Dixon et al., 2001). In our result, we found HuR binding specifically to the HOX11 mRNA 3'UTR, which suggests that HuR may bind and stabilize HOX11 mRNA. It may be involved in the deregulation of HOX11 in T-ALL.

Although the in vivo function of HuR in the regulation of HOX11 expression need to be clarified, HuR protein showed its effectiveness in study of HOX11-HuR interaction in vitro. The different migration rate of RNA-protein complex in the EMSA reflects the heterogeneity of the complex and may be caused by the aggregation of RNA-protein complex.

Further study of the functional aspects of HuR-HOX11 3'UTR interactions may provide information on the regulation of the gene in vivo. The HOX11 ARE or its mutations could be inserted downstream of reporter gene for the transfection of different cell types to examine the influence and effectiveness on the post-transcriptional regulation of reporter gene in vivo. This study would help to understand the critical structure features and the function of the ARE that may be involved in the HOX11 gene regulation at the post-transcriptional level. A further scanning of the HuR expression level and experimental
manipulation in T-ALL would also help to explore the possible role of HuR in the HOX11 deregulation in T-ALL.

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References


