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Yeast One-hybrid System Used to Identify the Binding Proteins for Rat Glutathione S-transferase P Enhancer I[1]

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Objective To detect the trans-factors specifically binding to the strong enhancer element (GPEI) in the upstream of rat glutathione S-transferase P (GST-P) gene. **Methods** Yeast one-hybrid system was used to screen rat lung MATCHMAKER cDNA library to identify potential trans-factors that can interact with core sequence of GPEI(cGPEI). Electrophoresis mobility shift assay (EMSA) was used to analyze the binding of trans-factors to cGPEI. **Results** cDNA fragments coding for the C-terminal part of the transcription factor c-Jun and rat adenine nucleotide translocator (ANT) were isolated. The binding of c-Jun and ANT to GPEI core sequence were confirmed. **Conclusions** Rat c-jun transcriptional factor and ANT may interact with cGPEI. They could play an important role in the induced expression of GST-P gene.

Key words: Glutathione S-transferase P enhancer I; Yeast one-hybrid system trans-action factor

INTRODUCTION

Rat glutathione S-transferase P (GST-P) which is absent from the normal hepatocytes, is specifically induced at an early stage of chemical hepatocarcinogenesis[1-4]. It appears to be a reliable marker for hepatocellular carcinogenesis in rat[5]. Transcriptional activation is a crucial step in inducing GST-P expression[6]. To understand the molecular mechanism of GST-P gene activation, it is important to explore the specific interaction between cis-active DNA elements and their trans-acting factors. So far as we know, there are multiple regulatory elements in the 5'-flanking sequence of the GST-P gene. Two enhancer elements, termed GPEI and GPEII, are located about -2.5 kilobases (kb) to -2.2 kilobases

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Abbreviatons:glutathione S-transferase P(GST-P); adenine nucleotide translocator(ANT); kilobases (kb); phorbol 12-O-tetradecanoate 13-acetate (TPA); core sequence of GPEI(cGPEI), electrophoresis mobility shift assay(EMSA).

Biography of the first author: Liao Ming-Xiang (1971-), female, main research field is transformation and regulation of rat glutathione S-transferases P gene.

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(kb) upstream from the cap site of this gene[7]. GPEI is a powerful enhancer region and a dominant element controlling GST-P expression. The core sequence of GPEI consists of a palindromic dyad of TRE-like sequence motifs[8]. The trans-acting factors binding to GPEI need to be clarified. In the present study, we used yeast one-hybrid system to screen rat lung MATCHMAKER cDNA library to identify potential trans-factors that can interact with the core sequence of GPEI (cGPEI).

MATERIALS AND METHODS

Yeast One-Hybrid System

The screening procedure was essentially performed as described in the protocols PT1031-1 and PT1020-1 (Clontech Laboratories). Three tandem copies of cGPEI flanked by an EcoRI site and a XbaI and SalI sites were generated by annealing sense and antisense oligonucleotides. The core sequence of GPEI: 5-TTGCTGAATCATAGTGACTGACTA-3 and 3-AACGACTTAGTATCACTGACTG AT-5. These fragments were inserted into a multi-cloning site upstream of the promoter of HIS3 and LacZ reporter gene promoter to generate the target reporter plasmid pHISi-1-GPEI and pLacZi-GPEI. The target-reporter constructs were linearized and sequentially transformed into the yeast strain YM4271 (MATα ura3-52, his3-200, ade2-101, lys2-801, leu2-3, 112, trp1-903, tyr1-501) by using the lithium acetate method to obtain a reporter yeast stain YMGPE. The yeast was then transformed with rat lung MATCHMAKER cDNA library which was constructed into the leucine encoding pACT2 vector downstream of the GAL4 activation domain (Clontech) and selected on histidine, uracil and leucine deficient (SD/-His/-Ura/-Leu) plates containing 3-aminotriazole (15mmol/L was shown to be optimal for suppressing HIS3 background growth). Large colonies were transferred onto Whatman#5 filters and further screened for β-galactosidase activity. After being placed in liquid nitrogen for 30s, the filters were incubated in buffer containing 0.8 mmol/L 6-bromo-4-chloro-3-indolyl-β-D-galactosidase at 30℃. The positive interaction was determined by the appearance of blue colonies. The LacZ$^+$ colonies were selected and plasmids were recovered. The candidate plasmids isolated from the positive clones were transformed into YMGPE to retest for His$^+$ phenotype and β-galactosidase activity. Those that could reproduce the positive phenotypes were called true positive clones and their cDNA inserts were partly sequenced by using primers flanking the multi-cloning site of pACT2 vector: 5'-GATGATGAAGATACCCCACC-3'. Sequences were analyzed with Clone4.0 and compared with GenBank data base entries.

Electrophoretic Mobility Shift Assay (EMSA)

The double stranded oligonucleotide containing three tandem copies of cGPEI described as before was used as probe. Overhanging ends were labeled by using Klenow enzyme and [α-^{32}p] dATP. Nuclear extracts were prepared from yeast cells as described in the protocols PT1031-1 (Clontech Laboratories). EMSA was performed as described previously[9]. In the binding reaction, ^{32}P-end labeled probe (0.1-1ng, 10 000cpm) was mixed with 1-2 μg of poly(dI-dC), 2-6 μg of yeast protein extract in a 20 μl reaction mixture containing 10% glycerol, 10 mmol/L Na-HEPES PH7.9, 50 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 2.5 mmol/L Tris-HCl PH7.9. After incubated at 4℃ for 1 h, the mixture was resolved on 5% nondenaturing polyacrylamide gel. In the competition assay, competitors were added to the reaction mixture and incubated at 4℃ for 30 min prior to the addition of the probe.

RESULTS

Positive Clones Obtained From Rat Lung MATCHMAKER cDNA Library

A yeast one-hybrid system was employed to clone the factor binding to cGPEI from rat lung MATCHMAKER cDNA library. 5 positive clones were isolated. The five candidate plasmids isolated from the positive clones were transformed into YMGPE to retest for His^+ phenotype and β-galactosidase activity. Two of them that could reproduce the positive phenotypes were called true positive clones, named pYGPE1 and pYGPE2 (Fig. 1). After sequencing, pYGPE1 revealed sequence homology to the known cDNA of the transcription factor c-Jun. pYGPE2 sequence was homologous to the known cDNA of rat adenine nucleotide translocator (ANT).

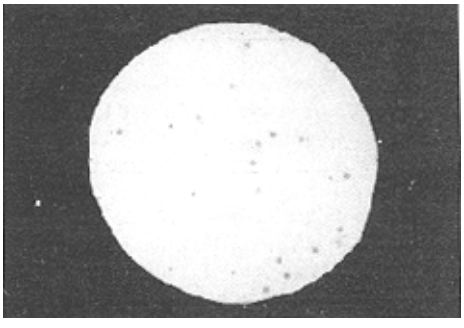

FIG. 1. Measuring results of the activity of LacZ gene in YMGPE strain transformation with the candidate plasmids isolated from positive clone. (The color of His^+LacZ^+ is blue.)

Identification of Positive Yeast Clone Lysates Interacting With cGPEI

The DNA-binding properties of two positive yeast clones (called YMGPE1 and YMGPE2) with EMSA were verified by using labeled oligonucleotides containing three tandem copies of cGPEI as a target. A retarded shift was generated with nuclear proteins from both of YMGPE1 and YMGPE2 (Figs. 2 and 3) containing the fusion protein of GAL4 activation domain and the C-terminal domain of c-Jun or ANT.

DISCUSSION

Muramatsu *et al.* found that AP1 contributed to GPEI-mediated transcription activity, but failed in sufficiently accounting for all of GPEI's enhancer capability. Rather, another transcription factor requiring the entire core sequence of GPEI for binding contributed to GPEI-mediated activation[10]. Comparably, results from other laboratories suggested that AP-1 were apparently not the prerequisites for GST-P expression, since GST-P was not always accompanied by appreciable c-Jun or c-Fos[11]. It remains to be clarified what factors are actually responsible for the expression of GST-P in rat hepatic preneoplastic lesions or normal tissues.

ucGPE I (folds)	0	0	0	0	0	100	300
ucGPE II (folds)	0	0	100	200	300	0	0
Lane	1	2	3	4	5	6	7

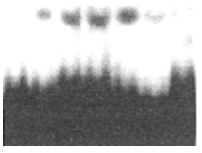

FIG. 2. Specific binding pattern of protein extracts from YMGPE1 with cGPEI probe. The molar excess folds of unlabeled oligonucleotides are indicated above each lane. Lane 1: free probe. ucGPEI: unlabeled GPEI core sequence (specific competitor); ucGPEII: unlabeled GPEII (nonspecific competitor).

Our previous study has revealed that chemical stimulation by phorbol 12-O-tetradecanoate 13-acetate (TPA) on GST-P expression was mainly through GPEI and some proteins could specifically bind to cGPEI[9,12]. cGPEI contains TRE-like sequence, and it is speculated that the induced expression of GST-P is due to the binding of AP-1 or the corresponding factors to cGPEI induced by TPA. The previous results of *in vitro* assay indicated that c-Jun may be the protein binding to GPEI to induce the expression of GST-P.

ucGPE I (folds)	0	0	0	0	100	300
ucGPE II (folds)	0	100	200	300	0	0
Lane	1	2	3	4	5	6

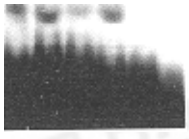

FIG. 3. Specific binding pattern of protein extracts from YMGPE2 with cGPEI probe. The molar excess folds of unlabeled oligonucleotides are indicated above each lane. Lane 1: free probe. ucGPEI: unlabeled GPEI core sequence (specific competitor); ucGPEII: unlabeled GPEII (nonspecific competitor).

In the present study, we identified that oncogene product c-Jun binds to the core sequence of GPEI in yeast cells. Since the detection of DNA-protein interaction occurs while proteins are in their native configurations in yeast cells, the one-hybrid system offers maximum sensitivity on the regulation of eukaryotic gene. Furthermore the present study found that the domain formed by 171st-334th amino acids of c-Jun in rat plays an important role in the interaction of c-Jun and cGPEI, which is consistent with the previous reports[11,13].

Our results indicated that rat ANT could specifically bind to cGPEI. Rat ANT is an integral protein localized in the mitochondrial inner membrane, which catalyzes the exchange of adenine nucleotides across the bilayer. The gene that encodes ANT has been reported to be located in chromosome. Early studies on the biogenesis of ANT provided the first demonstration that mitochondrial precursor protein pools were present in the cytoplasm[14]. While there are few reports about the involvement of ANT in the regulation of gene expression as transcription factor, what role ANT plays in the regulation of gene expression remains to be clarified.

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