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Molecular Cloning of a Novel Rat Salt-Tolerant Protein by Functional Complementation in Yeast

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To elucidate the genetic basis of salt-sensitivity in mammalian hypertension, we isolated six rat complementary DNAs by functional complementation in yeast. These genes were able to substitute for the salt-tolerant activity of *HAL1* which confers salt tolerance by modulating the cation transport system in yeast. We identified these genes as β-globin, λ-crystallin, androgen-regulated protein, mitochondrial cytochrome b, a homologue of infant brain cDNA, and a novel gene, called salt-tolerant protein (STP). STP contains 1964 bp nucleotides and an open reading frame which encodes 496 amino acid residues. Northern blot analysis showed that STP mRNA is expressed in various rat tissues.

Essential hypertension is a multifactorial disease which is complicated by genetic and environmental factors. Excessive dietary sodium intake is an important factor in the hypertension. Several studies have suggested that salt sensitivity is affected by age, race, and the renin angiotensin and sympathetic nervous systems. Although the molecular basis of the salt sensitivity in hypertension is not yet fully understood, several biochemical alterations have been documented in hypertensive rats and humans. Weinberger et al. (1) reported an association between the haptoglobin phenotype and the sodium-sensitivity and -resistance of blood pressure. Dahl's salt-resistant rat has a mutation in cytochrome P450(11β) (2). In a linkage analysis, nerve growth factor receptor (3), S_A gene (4,5) and angiotensin converting enzyme (6) were linked to salt-sensitive hypertension. Furthermore, gene targeting has demonstrated that atrial natriuretic factor (7) and guanylyl cyclase A receptor (8) modulate the blood pressure response to dietary salt.

In 1992, Gaxiola et al. (9) isolated a novel gene, *HAL1*, in the yeast *Saccharomyces cervisiae* which improved salt tolerance by modulating the cation transport system. Previous workers have cloned mammalian genes by using a biological screen in yeast (10-12). We believe that this system is generally useful for cloning genes from higher eukaryotes when functional expression is possible in yeast.

In the present study, to elucidate the genetic factors involved in rat salt sensitivity, we screened a rat cDNA expression library using functional complementation to improve the growth of the yeast *S.cervisiae HAL1* null strain.

MATERIALS AND METHODS

Materials. cDNA synthesis and sequencing kits were obtained from Stratagene (La Jolla, CA) and United States Biochemicals (Cleveland, OH). Yeast expression vector, Lambda Max1 and pYEUra3 was from Clontech Laboratories

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The nucleotide sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank data base (accession number D50557) for salt-tolerant protein (STP).

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(Palo Alto, CA). Various DNA-modifying enzymes and endonucleases were purchased from Takara Shuzo (Kyoto, Japan) and Nippon Gene (Toyama, Japan).

HAL1 null strain construction. The *HAL1* gene from a *S.cervisiae* genomic library was isolated by PCR and then subcloned into pUC13. The *HAL1* gene was disrupted by interrupting the coding region with a selectable marker histidine gene and transformed into the parent strain DY150. Transformants were selected with-HIS, high salt (1.4M NaCl) media.

Cloning and sequencing. A poly(A)$^+$ RNA fraction was prepared from Wistar rat kidney and used to construct a cDNA library in λMax1, which was converted into the plasmid vector pYEUra3 by a helper phage. The cDNA library was screened by functional complementation of a null mutation of the yeast *HAL1*. About 1×10^4 transformants were screened and 12 well-grown colonies were obtained in solid media which contained 1.4M NaCl and lacked both uracil and histidine. After retesting, seven clones were obtained and these were then partially or completely sequenced by the dideoxynucleotide chain termination method (13) using a Sequenase DNA-sequencing kit.

Halotolerance test. Before halotolerance tests (14), cells were grown for 2 days to saturation (absorbance at 660nm ~2.7). For testing in solid media, the saturated cultures were diluted 50-fold with water and 3 μl were dropped on normal medium and on medium supplemented with 1.4M NaCl.

Northern blot analysis. Total and poly(A)$^+$ RNA were prepared from various rat tissues by the guanidine thiocyanate method. Twenty micrograms of total RNA and 2 micrograms of poly(A)$^+$ RNA were denatured with 50% formamide/ 6.5% formaldehyde and electrophoresed on 1.2% agarose gels containing 6% formaldehyde. The separated RNA was transferred to a Hybond^{TM}-N nylon membrane and hybridized with ^{32}P-labeled full-length STP cDNA and a human G3PDH probe.

RESULTS AND DISCUSSION

To elucidate the genetic factors involved in salt tolerance in the rat, we isolated rat cDNAs that could complement the salt-tolerant activity of the yeast disrupted *HAL1* gene. *HAL1* gene was isolated from a yeast genomic library by PCR and subcloned into pUC13. The *HAL1* gene had been disrupted by insertion of the histidine gene at an *Afl*II site. *HAL1* disrupted plasmid was transformed into the yeast parent strain DY150. Homologous recombination occurred between the transformed *HAL1*::HIS3 region and the original yeast chromosomal *HAL1* gene. Transformants were screened by a simultaneous double selection for their growth. First, we selected histidine-positive clones that grew well in medium that lacked histidine. Next, we selected a *HAL1*-deficient strain whose growth was decreased in medium containing 1.4 M sodium chloride.

Poly(A)$^+$ RNA fractions were prepared from Wistar rat kidney and used to construct a cDNA expression library in λMax1 (*Xba*I and *Xho*I site), which was converted into the plasmid vector pYEUra3 by a helper phage. pYEUra3 carries a selectable marker gene uracil. The rat kidney cDNA expression library was then transformed into the yeast *HAL1*-deficient strain. We screened approximately 1×10^4 transformants and selected 12 well-grown colonies in solid media containing 1.4M NaCl and lacking uracil and histidine. After retesting, seven well-grown colonies were obtained. Seven of the 12 transformants improved the growth of the yeast *HAL1*-deficient strain in media with a high concentration (1.4M) of NaCl, but had no

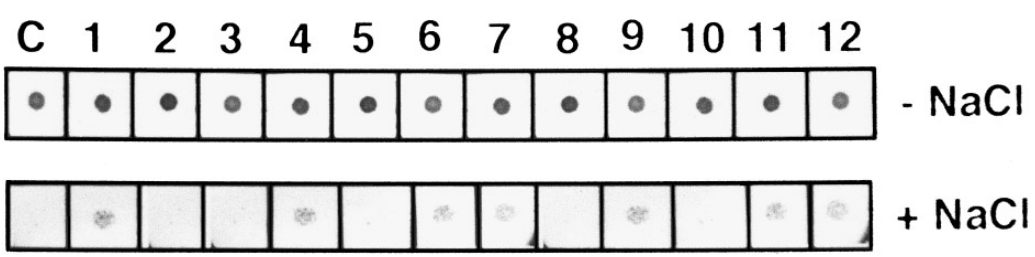

FIG. 1. Screening (halotolerance test). Yeast strain DY150 disrupted *HAL1* gene was transformed with 12 unknown cDNA plasmids. Saturated cultures (absorbance at 660 nm ~2.7) from transformants of each plasmid were diluted 50-fold with water and 3.0 ml was dropped on normal solid medium (−NaCl) and on medium supplemented with 1.4M NaCl (+NaCl). C represents control vector pYEUra3 transformant.

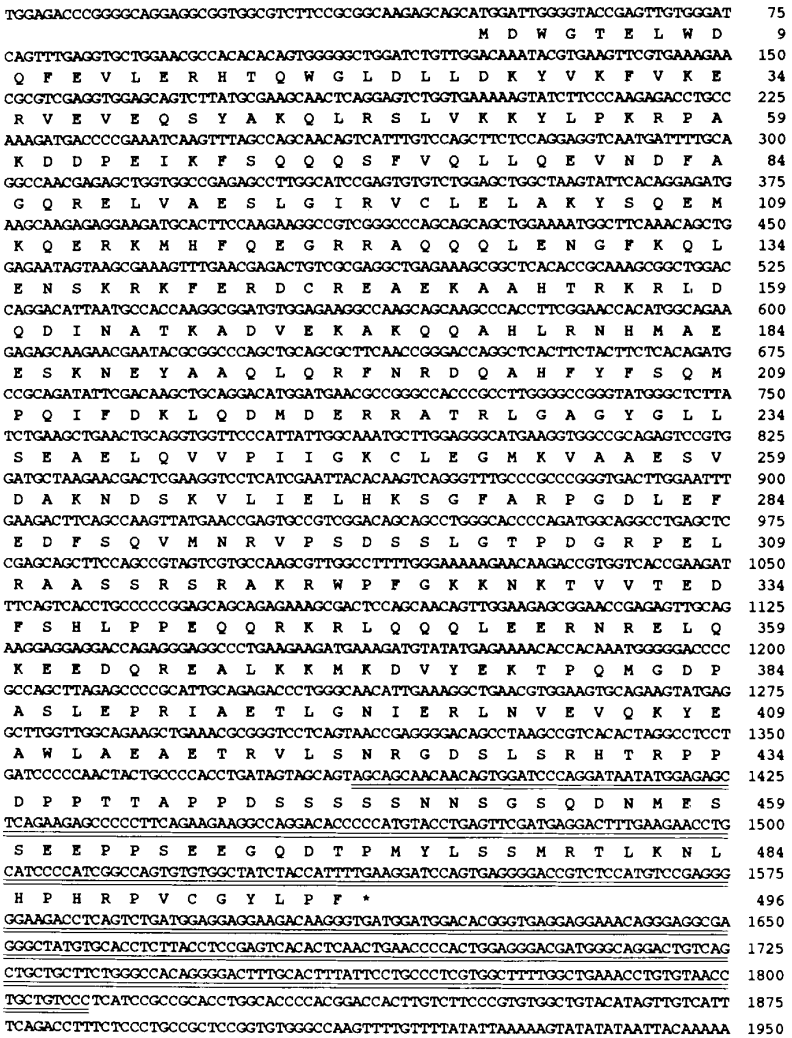

FIG. 2. Nucleotide and deduced amino acid sequences of salt-tolerant protein (STP). The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. Nucleotides and amino acids are numbered on the right. The stop codon is indicated by an asterisk. Double underlining denotes a nucleotide sequence that shows partial homology to the nucleotide sequence of human thyroid receptor-interacting protein 10 (Trip 10).

effect in normal media (Halotolerance test, Fig.1). The growth of these colonies in high-salt media means that these transformed genes can complement the salt-tolerant activity of the *HAL1* gene. Seven clones were partially sequenced and six cDNAs were obtained. One of these showed no significant similarity to any known proteins with regard to the amino acid sequence and was registered as salt-tolerant protein (STP). Finally, we isolated six clones that were identified as β-globin, λ-crystallin, androgen-regulated protein, mitochondrial cytochrome b, a homologue of infant brain cDNA and the novel gene STP. There is no sequence homology between *HAL1* and these cDNAs. STP is predicted to encode a protein of 496 amino acid residues with a calculated mass of 57 kDa (Fig.2). In a Northern blot analysis, STP was shown to be expressed in several different rat tissues as a single transcript of 2.1 Kb (Fig.3).

Serrano et al. have isolated two yeast genes, *HAL1* (9) and *HAL2* (14), that improve yeast

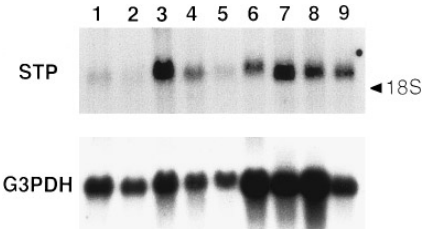

FIG. 3. STP mRNA in various rat tissues. Total RNA and poly(A)$^+$ RNA were prepared from various rat tissues by the guanidine thiocyanate method. Twenty micrograms of total RNA from the ventricle (lane 1), atrium (lane 2), aorta (lane 3), adrenal gland (lane 4), liver (lane 5), and skeletal muscle (lane 6) and 2.0 μg of poly(A)$^+$ RNA from the kidney (lane 7), brain (lane 8), and spleen (lane 9) were subjected to Northern blot analysis. The position of 18S ribosomal RNA is indicated on the right.

growth under salt stress when overexpressed. The *HAL2* gene is identical to the yeast methionine biosynthetic gene *MET22* and the encoded protein is homologous to bovine inositol phosphatases (14,15). *HAL1* confers salt tolerance by modulating cation transport systems and is conserved in yeast and plants. The growth advantage obtained by overexpression of *HAL1* is specific for NaCl stress. The most important feature detected in yeast cells overexpressing *HAL1* is the increased accumulation of K^+ in high NaCl medium. The *HAL1* protein could interact with transport systems which determine intracellular K^+ homeostasis (9). From these results, the mechanism of the salt-tolerant activity conferred by these isolated genes has been speculated to involve Na^+ and/or K^+ transport systems. STP shows a particularly high degree (78-95%) of nucleotide sequence identity to human thyroid hormone receptor-interacting protein 10 (Trip10) mRNA at the 3′ end and the non-coding region (Fig.2, double underlining). Lee et al. reported only C terminal nucleotide and amino acid sequences (16). We suppose that STP and Trip10 are translated from different reading frames. Consequently, there is less similarity in the peptide sequence (20%). We suggest that STP may be homologue of Trip10. Thyroid hormone receptors are hormone-dependent transcription factors that regulate the expression of specific target genes. These findings demonstrate that STP may be involved in a hormone-dependent transcriptional pathway.

In conclusion, we isolated novel and known rat cDNAs that confer salt tolerance, using functional complementation to improve the growth of the yeast *S.cervisiae HAL1*-deficient strain under high salt conditions. Further studies are needed to demonstrate the function of these proteins at the genetic and biochemical levels.

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