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Site-Directed Saturation Mutagenesis of Yeast Gcn4p at Codon 242

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Abstract Gcn4p, a transcriptional activator protein of the yeast, *Sacchromyces cerevisiae*, binds to the specific sequence in the promoters of many amino acid biosynthetic genes for general control. The serine residue (Ser 242) of Gcn4p directly contacts the DNA. Here, for inspecting the DNA binding properties and the level of transcriptional activation of Gcn4p, we introduced a polymerase chain reaction (PCR) site-directed saturation mutation library into the Ser 242 site using 2 outside primers and 2 oligonucleotides with its codons fully degenerated. The sequencing analysis of 146 samples revealed the even nucleotide distribution within the experimental error showing 23, 26, 25, and 26% frequency of U, C, A, and G bases, respectively. This method turned out to be a simple, fast, and economical method for constructing a library of all 20 amino acids at specific codon.

Key words: Mutagenesis, polymerase chain reaction, Gcn4p, *Saccharomyces cerevisiae*

The development of the polymerase chain reaction, (PCR) [17] has led to simple and fast methods for site-directed mutagenesis [7]. To direct the synthesis of mutant DNA, mismatches were introduced into the primers. Complementary oligonucleotide primers and the polymerase chain reactions have been used to generate two DNA fragments having overlapping ends. These fragments were combined in a subsequent reaction in which the overlapping ends annealed, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting product was amplified further by PCR. Specific alterations in the nucleotide sequence could be introduced by incorporating nucleotide changes into the overlapping primers. Insertion and deletion mutations could be generated by this method [4].

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During the amino acid starvation, expression of many yeast amino acid biosynthetic genes depends on a transcriptional activator, Gcn4p [3, 5], a member of the family of bZIP proteins. Gcn4p is a yeast protein that binds to the promoters of many amino acid biosynthetic genes and coordinately activates their transcription. It is a 281 amino-acid protein and binds to the AP-1 binding site (ATGACTCAT) as a dimer. The DNA binding specificity of Gcn4p resides in its basic region, the C-terminal 60 amino acids [5]. The basic region directly binds to DNA [1], while dimerization is mediated by the leucine zipper [12, 14, 20]. The DNA binding domain of Gcn4p shows about 45% sequence identity to the Jun oncoprotein.

AP-1 proteins, human transcription activating factors, recognize the same binding sites as Gcn4p. The ATF/CRE site is recognized by a family of proteins referred to as ATF (activating transcription factor) or CREB (c-AMP responsive element binding). AP-1 and ATF/CREB proteins are structurally related and recognize identical half-sites (TGAC), but they differ in their requirements for half-site spacing [9, 10].

With the advent of the PCR, it has been possible to introduce a series of mutations into the specific DNA region [6, 7, 8]. Of the basic DNA binding domain, the serine residue of Gcn4p (Ser 242) is critical for contacting the central base pair(s) of the target sites [11]. To address how different amino acid replacements at this site affects the binding and activation properties of Gcn4p, we first prepared all the possible amino acid replacements at the Ser 242 site by utilizing the site-directed saturation mutagenesis.

Bacterial Strain and Plasmids

Escherichia coli XL1-Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ*Δ M15 Tn*10* (*tet*^{r})]) was used in cloning. YCp88-Sc4400 [15, 16], a centromeric vector expressing full-length Gcn4p, was used as a plasmid template for the first PCR and cloning

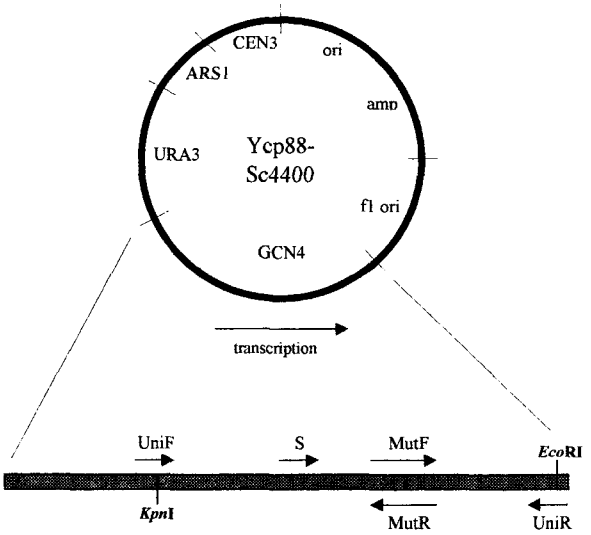

Fig. 1. Structure of YCp88-Sc4400 indicating the locations of important genetic elements on the vector and Gcn4p coding region. Primer annealing sites are indicated by arrows.
S, a primer for sequencing; *, the target region of mutation (Ser242).

vehicle. The map of Ycp88-Sc4400 is shown in Fig. 1 with primer annealing sites.

Primers

Two oligonucleotides containing restriction sites for cloning were used as universal primers for PCRs (UniF: 5'-AAGTTTCTCTGGTACCAT-3', UniR, 5'-ACGAATTC-GAGCTCATTAA-3'). Two complementary oligonucleotides contained a randomized codon (NNN) with 12 flanked bases on each side of the randomized area and were used as mutagenic primers (MutR: 3'-CGACGCGCAGCAN-NNGCACGAGCATTT-5', MutF: 5'-GCTGCGCGTCG-TNNNCGTGCTCGTAAA-3'). MutR and MutF were complementary with each other except the randomized codon. An oligonucleotide used as sequencing primer was complementary with the template strand between the forward universal primer and mutagenic primers.

The First PCR

PCRs were carried out using *Taq* polymerase as specified by the manufacturer (Promega). Briefly, amplification of DNA fragments from the plasmid template was achieved by adding 5 ng of wild-type plasmid DNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 4.0 mM $MgCl_2$, 200 μM each of dNTP, 1 pM of one universal primer (UniF or UniR) and one mutagenic primer (MutR or MutF), and 2.0 units of *Taq* polymerase in a final volume of 100 μl. This mixture was overlaid with 100 μl of mineral oil and subjected to 30 cycles of denaturation (45 sec, 94°C), annealing (45 sec, 52°C), and extension (90 sec, 72°C) using a DNA Thermal Cycler (Perkin Elmer Cetus, Emeryvill, U.S.A.). The products of the reaction were analyzed on

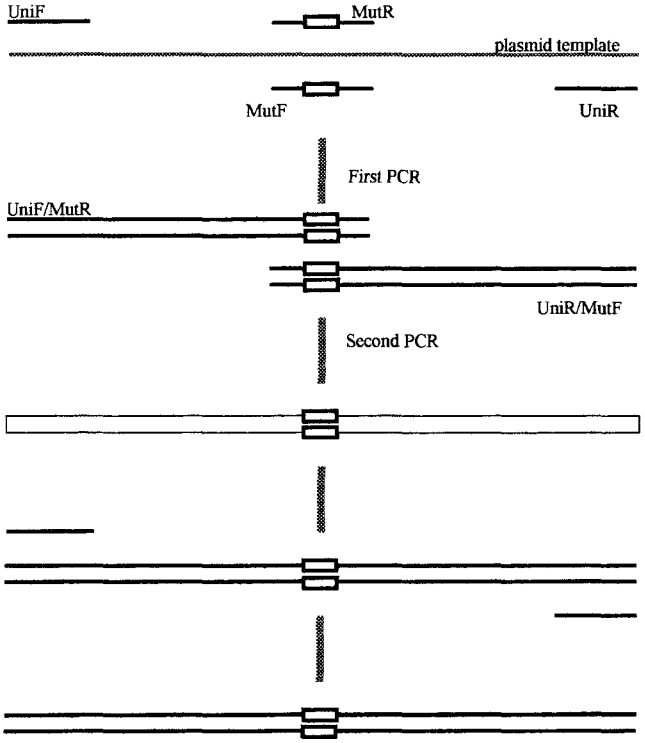

Fig. 2. Schematic presentation of the two-step PCR mutagenesis method.
In the first PCR, the mutagenic primers (MutR and MutF) and the universal primers (UniF and UniR) are used yielding two products, which after an annealing and extension step are taken as templates in the second PCR. The site of mutagenesis is indicated by the small rectangle.

an agarose gel containing 1.5% agarose (Sigma co., St. Louis, U.S.A.) and 0.5 μg ethidium bromide/ml in Tris-acetate buffer. Electroelution of the first PCR products were performed as described by Sambrook *et al.* [18].

The Second PCR

The second PCR reaction volume was 100 μl containing 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 4×10^{-3} pmol of the purified first PCR products (UniF/MutR and UniR/MutF). The reaction mixture was heated for 5 min at 94°C to denature the DNA completely, and then cooled slowly to 37°C for 1 h to anneal the DNA at the complementary region. 2.0 units of *Taq* polymerase and 200 μM dNTP were added to the mixture, and extension was carried out once at 72°C for 10 min. 0.4 pM of each universal primer was then added and the reaction mixture was subjected to 35 cycles of denaturation (45 sec, 94°C), annealing (45 sec, 52°C), and extension (90 sec, 72°C). Final extension reaction was performed at 72°C for 10 min.

Cloning and Sequencing of the Mutated GCN4 Genes

The mutagenic fragment and plasmid DNA were digested with two restriction endonucleases (*Eco*RI and

*Kpn*I, Boehringer Mannheim, Germany). 5'-phosphate was removed from the digested plasmid DNA to prevent self-ligation. The dephosphorylated plasmid DNA was mixed with an equimolar amount of mutagenic DNA fragment. Ligation reaction contained 1 unit of T4 DNA ligase (Promega, Madison, U.S.A.) and 0.1 volumes of 10× ligation buffer (300 mM Tris-HCl, (pH 7.8); 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP). The mixture was incubated for 16 h at 16°C. XL1-Blue competent cells were transformed with the ligation mixture and cultured in LB medium containing 50 μg/ml ampicillin. Nucleotide sequences were determined by the dideoxy-chain termination method of Sanger *et al.* [19] using the Sequenase Version 2.0 DNA sequencing kit (United States Biomedicals, Cleveland, U.S.A.) and analyzed by polyacrylamide gel electrophoresis.

Detection of Codons and Amino Acids from the PCR Product

In separate PCRs (the first PCRs), two fragments of the target gene sequence were amplified. Each reaction used one universal primer (UniF and UniR) that hybridized at one end of the target sequence and one internal mutagenic primer (MutR and MutF) that hybridized at the site of mutation and contained the mismatched bases. The two fragments (UniF/MutR and UniR/MutF), generated in the first PCR, could be fused by denaturing and annealing them in a subsequent primer extension reaction. The overlap allowed one strand from each fragment to act as a primer on the other, and extension of this overlap resulted in the mutant product. Even though the annealing of the short overlap between the two fragments might occur at low frequency, the further inclusion of additional universal primers allowed the final mutant product to be amplified by PCR. The first PCR and the second PCR products were analyzed on 1.5% agarose gel. 146 clones were available for sequencing reactions and the results were shown in Table 1.

56 codons were detected among the possible 64 codons and these encoded 19 amino acids and 3 stop codons (Table 1A). The occurring frequency at the first base position within the Ser 242 site was 25% of U, 21% of C, 27% of A, and 27% of G. That of the second base position was shown to be U base with 18%, C base with 29%, A base with 23%, and G base with 30%. That of third base position was U base with 24%, C base with 28%, A base with 27%, and G base with 21%. Totally, the occurring frequency at all the three positions was U base with 23%, C base with 26%, A base with 25%, and G base with 26% (Table 1B). The AUG codon for methionine was not found, probably due to the low probabilities of U in the second base position and G in the third base position.

PCR has been employed in site-directed mutagenesis. A few general methods for mutagenesis using two-step PCR have been introduced [3, 5, 7, 14]. The method described in this report is a modification of the overlap extension method [5] and basically has similar experimental design to other published methods.

Table 1. Diagram showing the frequencies of occurring bases at each position in codons.

(A)

1st base \ 2nd base	U			C			A			G		
U	UUU	F	4	UCU	S		UAU	Y		UGU	C	5
	UUC	F	2	UCC	S	2	UAC	Y	4	UGC	C	4
	UUA	L		UCA	S	3	UAA	STOP	4	UGA	STOP	2
	UUG	L	1	UCG	S	2	UAG	STOP	3	UGG	W	2
C	CUU	L		CCU	P	2	CAU	H	2	CGU	R	1
	CUC	L	1	CCC	P	5	CAC	H	2	CGC	R	1
	CUA	L	3	CCA	P	4	CAA	Q		CGA	R	3
	CUG	L		CCG	P	2	CAG	Q	1	CGG	R	4
A	AUU	I	1	ACU	T	1	AAU	N	1	AGU	S	5
	AUC	I	2	ACC	T	2	AAC	N	5	AGC	S	4
	AUA	I	3	ACA	T	3	AAA	K	2	AGA	R	
	AUG	M		ACG	T	3	AAG	K	4	AGG	R	3
G	GUU	V	3	GCU	A	4	GAU	D	1	GGU	G	5
	GUC	V	1	GCC	A	4	GAC	D	1	GGC	G	1
	GUA	V	3	GCA	A	6	GAA	E	2	GGA	G	1
	GUG	V	3	GCG	A	1	GAG	E	1	GGG	G	2

(B)

	U	C	A	G
1st	25%	21%	27%	27%
2nd	18%	29%	235	29%
3rd	24%	28%	27%	21%
sum	23%	26%	25%	26%

In this report, novel mutagenic oligonucleotides containing a randomized target codon were used for saturation mutagenesis. Only one set of reaction was needed to prepare the mutant library of interest. This reduces the time and effort required to generate site-directed mutagenesis. Although the described mutagenesis procedure required only two mutagenic primers and two universal primers, it allowed the easy construction of various mutants with high efficiency. To mutate another region of the target DNA, each mutation requires only two additional mutagenic primers. Therefore, this method is relatively simple, fast, and economical to use for constructing a library of all 20 amino acids at a specific site using only 4 primers.

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