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Arg243, Invariably Critical for the Transcriptional Activation of Yeast Gcn4p

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The arginine residue at position 243 (Arg 243) of the yeast transcription factor, Gcn4p, is invariably conserved among bZIP transcription factors. Using site-directed oligonucleotide saturation mutagenesis involving two-step polymerase chain reaction (PCR) amplification, random mutations were successfully introduced at the codon of 243 in the basic domain of Gcn4p. This mutant library was transformed into Gcn4p defective yeast strain and selected for the transcriptionally active colonies. All colonies which were transcriptionally active had arginines in the codon 243. In this study, the strand preference by Taq polymerase during mutagenesis was also tested. Oligonucleotides were specially designed to test whether or not the polymerase was preferred using the strand as a template. A population of randomly mutated products were cloned into an appropriate vector and characterized by DNA sequencing analysis. Saturation mutagenesis which was performed efficiently by this method revealed a strong bias in terms of strand preference of Taq polymerase by an approximate ratio of 3 to 1 in this study.

Key words: Mutagenesis, two-step polymerase chain reaction, Arg243, Gcn4p

By utilizing mutagenesis, it has been shown that a protein's structure and its activity are related to each other (17). One approach for designing a mutation experiment is to search for mutant organisms that exhibit the phenotype of interest after classical mutagenesis. The mutations obtained by this strategy would then be characterized by genetic mapping, followed by cloning and sequencing the wild-type and mutant genes. Because a given phenotype is generated from a particular protein expression, a second approach is to introduce point mutations into multiple codons, through which enormous amount of different codons are induced to specify a particular protein (1).

The improvement of PCR techniques (14) made it possible to devise simple and fast methods for site-directed mutagenesis (7). Mismatches introduced into oligonucleotide primers generated mutant DNA. Recently, the site-directed saturation mutagenesis method using PCR was successfully applied to generate a mutation library on the specific codon of a transcription factor, Gcn4p, from *Saccharomyces cerevisiae* without any bias in terms of base distribution (11).

In yeast, many genes encoding amino acid biosynthetic enzymes are subjected to a common regulatory system called the general control of amino acid biosynthesis. The product of the regulatory gene, Gcn4p, is required for an increase in transcription of general control-regulated genes when yeast cells are grown under amino acid-starvation conditions (18).

Gcn4p binds specifically to the promoters of amino acid biosynthetic genes and coordinately induces their transcription. The specific DNA binding activity resides in the 60 C-terminal amino acids, a basic region of Gcn4p. The activation function appears to involve just 19 amino acids that are centrally located in an acidic region of Gcn4p. In addition to their functional separation, the DNA binding and transcriptional activation regions of the protein can be separated physically by elastase cleavage (4). It has been known that the arginine residue (Arg243) of Gcn4p is invariably conserved among bZIP transcription factors. Crystallography studies showed that this residue had direct contact with the central base pair(s) of the target sites (2, 9).

In this report, a saturation mutation library on codon 243 was made and analyzed by DNA sequencing. In addition, this library was transformed into the Gcn4p defective yeast. Transformed yeasts were then spread on 3-amino-1,2,4-triazole (AT) plates to test for the ability of transcriptional activation of mutant Gcn4ps. AT, a competitive inhibitor of a His3 gene product, blocks the histidine biosynthesis pathway in yeast (8). Thus, the AT drug is used to test the activation of Gcn4p, and only strains with functional

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GCN4p	KRARNTEAARRSRARKLQRMKQL
JUN	KRMRNRIAASKCRKRKLERIARL
YAP1p	RTAQNRAAQRAFRERKERKMKEL
PAP1p	RKAQNRAAQRAFRKRKEDHLKAL
Nrl	RTLKNRGYAQACRSKRLQQRRGL
FOS	RRERNKMAAAKCRNRRRELTDTL
ATF1	RLMKNREAARECRRKKKEYVKCL
CREB	RLMKNREAARECRRKKKEYVKCL

Fig. 1. Basic regions of several bZIP transcription factors. Indicated sequences correspond to Gcn4p residues 231-253. All the transcription factors show Arg (**R**) at residue 243.

Gcn4p activities grow in the AT containing medium.

Materials and Methods

Bacterial strain and plasmids

Escherichia coli DH5α (*supE*44 *Δac u169(ϕ80 lacz ΔM15) hsdR*17 *recA*1 *gyrA*46 *thi-1 relA*1) was used in cloning. YCp88-Sc4400 (12, 13), a centromeric vector expressing full-length GCN4, was used as a plasmid template for the first PCR and cloning vehicle. This plasmid has a URA3 used as a yeast selective marker. The map of Ycp88-Sc4400 was shown previously (11).

Primers

Two oligonucleotides containing a restriction site for cloning were used as universal primers for PCRs (UniF: 5′-AAGTTTCTCTGGTACCAT-3′, UniR, 5′-ACGAATTCGAGCTCATTAA-3′). Two complementary oligonucleotides with a randomized codon with 21 flanking bases on each side of the randomized area were used as mutagenic primers (MutR: 3′-TGTCTTCGACGCGCAGCATCG**NN(G/T)**CGAGCATTTGACGTCGCATAC-5′, MutF: 5′-ACAGAAGCTGCGCGTCGTAGC**NN(G/T)**GCTCGTAAACTGCAGCGTATG-3′). MutR and MutF were designed to have G or T in the third base to detect which mutagenic primer contributes to the mutation of the codon. Therefore, after the sequencing if the sequenced codon has a G or T in the third base, it indicates that MutR made a contribution to mutate the codon. If it has a C or A, MutF contributed to the mutation. An oligonucleotide used as a sequencing primer was complementary to the template strand between the forward universal primer and mutagenic primer (MutR) as shown in Fig 3.

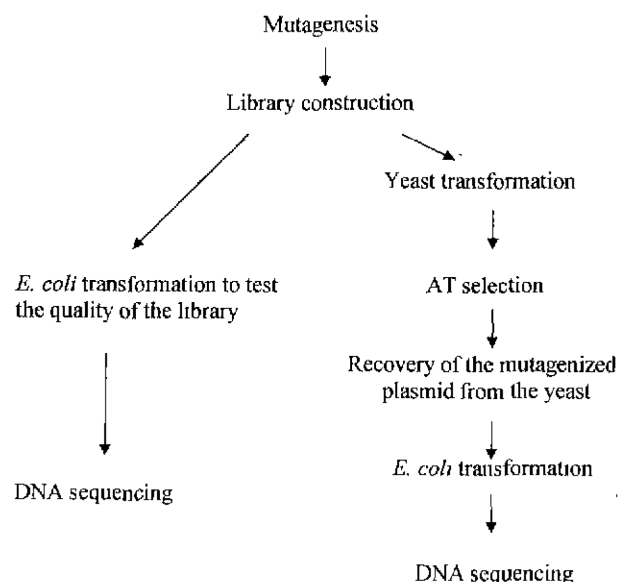

Fig. 2. Flowchart of the experiment performed in this report.

The first PCR

PCRs were carried out using *Taq* polymerase as

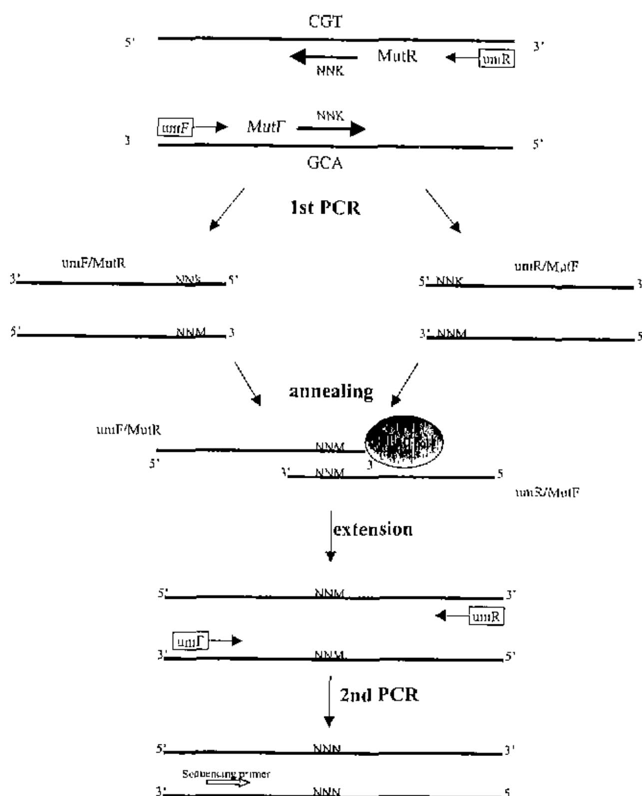

Fig. 3. Schematic presentation of the two-step PCR mutagensis method. In the first PCR, the mutagenic primer(MutR and MutF) and the universal primers (UniF and UniR) are used yielding two products, which after the annealing and extension step are used as a template in the second PCR.(N, any nucleotide; K, G or T; and M, A or C)

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, 10mM 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), along with 2.0 units of *Taq* polymerase in a final volume of 100 μl. These samples were 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). The products of the reaction were analyzed on an agarose gel containing 1.5% agarose (Sigma Co.) and 0.5 μg ethidium bromide/ml in Tris-acetate buffer/ 40 mM Tris-acetate, 1 mM EDTA (pH 8.0). Electroelution of the first PCR products was performed as described by Sambrook *et al.* (15).

Preparation of mutagenic DNA fragments by two-step PCR

In the first PCR, two fragments of the target gene sequence were amplified. The second PCR was performed as follows; the 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 first purified 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. During this procedure, two fragments from the first PCR products were hybridized at the site of mutation and contained mismatched bases. 2.0 units of *Taq* polymerase and 200 micromoles of dNTP were added to the mixture. The Extension reaction was performed at 72°C for 10 min. Therefore, the overlap allowed one strand from each fragment to act as a primer on the other, and the extension of this overlap resulted in the mutant product. Even though the annealing of the short overlap between the two fragments might occur at a low frequency, the inclusion of additional universal primers allowed the end mutant product that is formed to be amplified by PCR. Two picomoles of each universal primer was 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) (Fig 3).

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). The 5′-phosphate was removed from the digested plasmid DNA to prevent self-ligation. The dephosphorylated plasmid DNA was mixed with an equimolar amount of muta-genic DNA fragment. Ligation reaction contained 1 unit of T4 DNA ligase (Promega) and 0.1 vol. 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 at 16°C for 16 h. *E. coli* DH5α 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.* (16) using Sequenase Version 2.0 DNA sequencing kit (United States Biomedicals) and analyzed by polyacrylamide gel electrophoresis.

Yeast strain and procedure for transformation

Yeast *Saccharomyces cerevisiae* KY321a (*MATα*, *ura*3-52, *gcn*4::*TRP*1, *lys*2, *ade*2, *leu*2) was used and grown in YPD at 30°C. When $OD_{600\,nm}$ was close to 1.0, transformation was performed by the lithium chloride transformation protocol with 2 of mutant library DNA. These transformants were spread on synthetic complete (SC) plates with 30 mM AT lacking uracil and grown at 30°C for 3 days.

Yeast DNA minipreparation

Yeast clones that survived on a 30 mM AT plate were further grown in 5 ml of SC medium lacking uracil at 30°C for 2 days. Cells were collected by centrifuge at 14,000 rpm for 10 sec and the supernatant was discarded. Then, 200 μl of yeast lysis solution, 200 μl of phenol/chloroform/isoamylnalcohol and 0.3 g of acid-washed glass bead were added to these cell pellets, and vortexed vigorously for 2 min. After this mixture was centrifuged at 14,000 rpm for 10 min, the supernatant was transferred into a new tube and ethanol precipitation was performed. The pellet was dissolved in 100 μl of distilled water and added with 2 μl of RNase (10 μg/μl), then incubated at 37°C for 30 min. Finally, DNA was precipitated with ethanol and dissolved in 10 μl of distilled water. This DNA mixture was transformed into *E. coli* and sequenced.

Results and Discussion

Since PCR was devised, it has been employed in site-directed mutagenesis. A few general methods for mutagenesis using a two-step PCR have been introduced (3, 5, 6, 10). The method described in this report is a modification of the overlap extension method (5) and generally has a similar experimental design compared to other published methods.

In this report, novel mutagenic oligonucleotides containing a randomized target codon were used for sat-

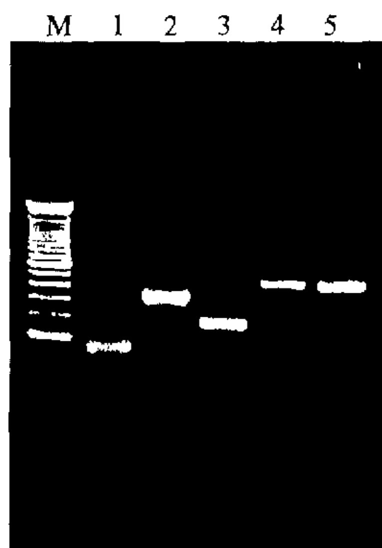

Fig. 4. Agarose gel (1.5%) electrophoresis analysis of the two-step PCR mutagenesis. M, 100 bp DNA ladder; lane 1, negative control; lanes 2 and 3, two products of the first PCR (UniF/MutR and UniR/MutF); lane 4, product of the second PCR (end product); and lane 5, positive control.

uration mutagenesis. Only one set of reaction was needed to prepare the mutant library of interest. This reduced 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 for the easy construction of various mutants with a high efficiency. In order to mutate another region of the target DNA, each mutation requires only two additional mutagenic primers. Therefore, this method is useful for introducing many mutations at various sites of the target DNA.

The intermediate and end products from the two-step PCR method were analyzed on 1.5% agarose gel (Fig 4) and the whole scheme of this experiment was shown in Fig 2.

Sequencing of the mutagenized DNA

30 clones were available for sequencing reactions and the results were shown in Table 1. 15 codons were detected 64 among possible codons, and 10 amino acids and 2 stop codons were encoded. The ratio of third bases, where the strand preference of the described method could be investigated, was 3 (C or A) : 1 (G or T) (23 clones : 7 clones). Also, the proportion of the first and second bases were analyzed in Table 2 (in the first base; G: 3%, A: 47%, T: 40%, C: 10%, in the second base; G: 20%, A: 37%, T: 37%, C: 6%). The proportion of G to T in the third base was 1 : 6 (1 clones : 6 clones), and the proportion of A to C was 10:1 (21 clones : 2 clones).

Table 1. Amino acids decoded from mutagenized DNA sequence at 243 site of Gcn4p

Amino Acid	Number of clones	Identified codons	Amino Acid	Number of clones	Identified codons
Ala	0		Leu	2	TTA CTG
Arg	5	AGA(4) CGA	Lys	4	AAA
Asn	1	AAC	Met	0	
Asp	0		Phe	5	TTT
Cys	0		Pro	1	CCA
Gln	0		Ser	0	
Glu	1	GAA	Thr	1	ACA
Gly	0		Trp	0	
His	0		Tyr	1	TAT
Ile	4	ATA(3) ATC	Val	0	
Stop	5	TAA(4) TGA	Total	30	15 codons

Strand preference of PCR mutagenesis

Clones were used for the sequencing reaction to investigate strand preference, and the results were shown in Tables 1 and 2. When two strands were used at the same rate as a template during the annealing step after the first PCR, the proportion of the third bases would be equal to all four nucleotides. However, our results revealed that the third bases were almost from the A/C strand. This data can be interpreted as a certain strand preference by Taq polymerase that may exist during PCR mutagenesis.

Taq polymerase may prefer to sit on one side of the annealed strand for reasons such as conformation, surrounding sequences, other positioning on the other side is less likely to happen for *Taq* polymerase probably due to the steric hindrance because the overlapping sequences are only 45 base pairs. Unfortunately, information regarding the base preference was not studied in this report. This phenomenon was not likely due to the annealing preference of the oligonucleotide primer to the template DNA because the annealing occured between the first PCR products and the first PCR product strand was preferred by Taq for extension, and the less hydrogen-bonded A or T bases appeared mostly after sequencing (Fig 3 and Table 2). Even though the mutant library used in this study was not large enough to assess the results sta-

Table 2. The proportion of each deoxynucleotide incorporated into the target codon after mutation.

	First codon	Second codon	Third codon
G	1 (3%)	6 (20%)	1 (3%)
A	14 (47%)	11 (37%)	21 (70%)
T	12 (40%)	11 (37%)	6 (20%)
C	3 (10%)	2 (6%)	2 (7%)
Total	30	30	30

tistically, A-T preference was clearly shown in the described results. Furthermore, we obtained interesting results in relation to third bases of the mutated region. If two mutagenic primers had a G or T in the on third position with an equal molar ratio, the third base of the mutated region could have G, A, T, C in the same ratio. However, the serious bias was detected (G : A : T : C = 1: 21: 6: 2) and the manufacturer could be suspected of making a mistake. However, the ratio of A or C to G or T could be explained with a possible existence of strand preference (3 : 1) by *Taq* polymerase in the polymerase chain reaction.

All AT resistant Gcn4p mutants have arginine at position 243.

3.2×10^3 clones of AT resistant GCN4 mutants were isolated out of 4×10^4 transformant clones. Plasmids in AT resistant clones were purified and sequenced in order to determine what kinds of amino acids can substitute 243Arg. 7 clones were used for sequencing, from which 6 clones were sequenced as AGAn(Arg) and one clone as CGAn(Arg). Due to the fact that the wild type codon 243 has CGT, it is very clear that these are all derived from mutagenized codons. The results confirmed that Arg at residue 243 of Gcn4p is extremely vital for the activation of transcription activation (Fig. 1). When Lys 243 mutant was transformed into GCN4 defective yeast in the presence of 40 nmM AT, no colony grew. This clearly indicates that no other amino acid residues at this position can substitute the arginine residue.

In this report, out of 64 possible codons, 15 codons were identified from the sequencing. If more sequencing had been done, all codons could have been found judging from the previous study (11). However, the purpose of the sequencing was to see if the library was properly constructed. We concluded that the library size of 4×10^4 was large enough to cover all 243 codons. Moreover, our data indicated that there was strand preference *Taq* polymerase for and 243 Arg was a critical residue for the activation of Gcn4p transcription.

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