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Upstream activation element of the *PH03* gene encoding for thiamine-repressible acid phosphatase in *Saccharomyces cerevisiae*

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The *PH03* gene of *Saccharomyces cerevisiae* encodes thiamine-repressible acid phosphatase and requires the positively acting regulatory protein TH12 for its expression. Deletion analysis of the 5'-flanking region of *PH03* gene revealed that an activating region located at nucleotide position −234 to −215 relative to the translation initiation codon is required for the expression and sensitivity to thiamine. A chemically synthesized DNA fragment covering −234 to −215 showed a significant level of expression when inserted in front of the *PH03* promoter lacking the activating region. Electrophoretic mobility shift assay demonstrated the presence of proteins that bound to the above DNA fragment in the nuclear extract from cells grown in thiamine-free medium. These findings suggested that this region between −234 and −215 acts as an upstream activation element of the *PH03* gene that can interact with regulatory proteins.

Thiamine-repressible acid phosphatase; Upstream activation element; *PH03*; *Saccharomyces cerevisiae*

1. INTRODUCTION

The *PH03* gene of *Saccharomyces cerevisiae* encodes thiamine-repressible acid phosphatase (T-rAPase) [1], and its expression is regulated at the transcriptional level by exogenous thiamine [2]. Recently, we isolated the *TH12* gene of *S. cerevisiae* [3], the transcript of which positively regulates the coordinated expression of T-rAPase and enzymes synthesizing thiamine monophosphate from 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole [4]. On the other hand, the expression of thiamine transport, T-rAPase and the enzymes for thiamine synthesis is controlled negatively by the intracellular level of thiamine pyrophosphate [5]. Thus, the yeast genes encoding the enzymes involved in thiamine metabolism are likely to share a common control mechanism. Every yeast gene contains an upstream activating sequence (UAS) located upstream of the TATA box. This sequence plays a major role in the transcription and regulation of the gene, and in several cases the binding of positive *trans*-acting regulatory factors has been demonstrated for these sites [6,7]. Some genes contain a different type of regulatory sequence for negative regulation. Since genes subject to a common control mechanism contain upstream elements that are similar in DNA sequence, it is necessary to identify the structure of such *cis*-acting sequences involved in the expression of the genes described above in order to elucidate the regulation of thiamine metabolism in yeast at the molecular level.

In this study, using DNA deletions, we localize the region responsible for the transcriptional activation of the *PH03* gene in response to the concentration of thiamine in the growth medium. Also, electrophoretic mobility shift assay demonstrated the occurrence of proteins capable of binding to the above region in nuclear extracts from cells grown in thiamine-free medium.

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2. MATERIALS AND METHODS

2.1. *Plasmids, enzymes and chemicals*

Plasmid pAP20, containing a 7.9-kbp yeast DNA fragment carrying the *PH05*, a structural gene for phosphate-repressible acid phosphatase, and *PH03* genes [8], as well as pRS315 [9], were supplied by Y. Kaneko (Institute for Fermentation, Osaka, Japan). Restriction enzymes were purchased from Nippon Gene (Toyama, Japan) and Takara Shuzo (Kyoto, Japan) and Toyobo Co. (Osaka, Japan). [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). All other chemicals were purchased from commercial suppliers.

2.2. *Strains, culture and transformation*

The yeast strain ND2-1C (*MAT pho3-1 pho5-1 leu2-3,112*), constructed in this study, was the host strain for transformation. X2180-1A (*MATa SUC2 mal gal2 CUP1*) was also used in this study. Yeast cells were cultured at 30°C in YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in a defined medium containing 0.67% yeast-nitrogen base (Difco Laboratories) supplemented with essential amino acids or Wickerham's synthetic medium supplemented with essential amino acids with or without thiamine [10]. Yeast cells were transformed by lithium acetate as described by Ito et al. [11].

Escherichia coli strains, DH5 and MV1184, were cultured in LB medium (0.5% yeast extract, 1% Bacto-Peptone, 1% NaCl, 0.2% glucose) at 37°C. When necessary, the medium was supplemented with

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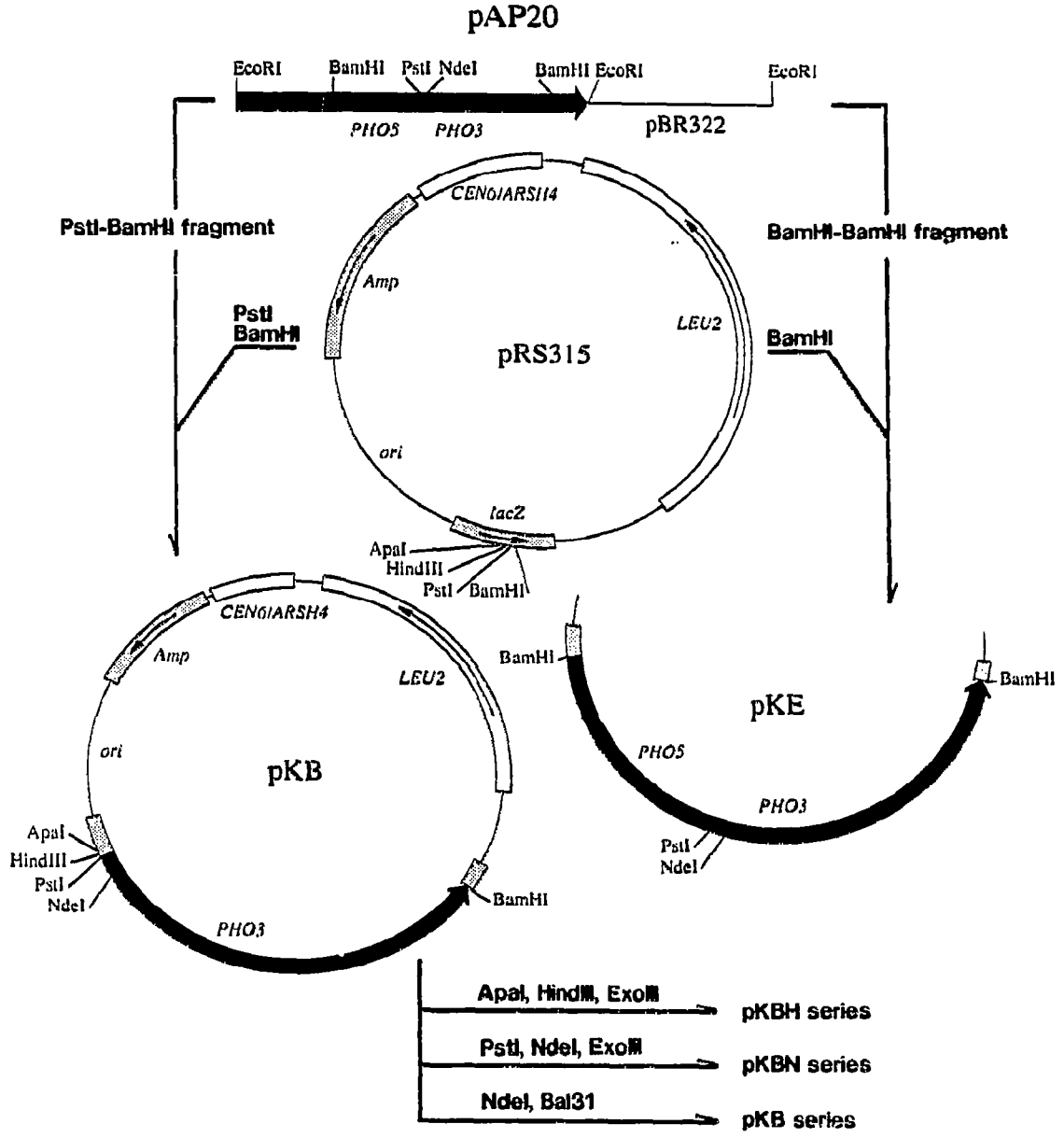

Fig. 1. Construction and structure of the plasmids used for analysis of the regulatory region of *PHO3*. Their sources and detailed procedures are described in Materials and Methods.

ampicillin (20 μg/ml). Bacterial transformations and preparation of plasmids by alkaline lysis were performed as described by Maniatis et al. [12].

2.3. *Plasmid construction*

The construction of plasmids used for the analysis of the *PHO3* regulatory regions is diagrammatically shown in Fig. 1. Plasmid pKE and pKB were constructed by cloning the 5.3-kbp *Bam*HI fragment containing both the *PHO5* and *PHO3* genes and the 3.3-kpb *Pst*I/*Bam*HI fragment containing the *PHO3* gene into pRS315, respectively. To create sequential and 5′-deletions in the *PHO3* upstream region, pKB was double-restricted with both *Apa*I and *Hin*dIII, and digested with exonuclease III (Promega, Madison, WI) for various periods at 22°C (pKBH series). The pKBN series was also constructed by the above procedure from pKB restricted with both *Pst*I and *Nde*I. To create internal deletions, pKB was cleaved at the *Nde*I site, treated with *Bal*31 nuclease-S (Takara Shuzo) for various periods at 22°C, filled in with Klenow fragment, and circularized (pKB series). The deletion ends were determined by direct dideoxy nucleotide sequencing from the plasmid DNA using a synthesized oligonucleotide as a primer [13].

2.4. *Reconstitution of the upstream activation element*

Synthetic single-stranded oligonucleotides containing putative upstream activation regions of *PHO3* flanked by *Hin*dIII linkers on both sides were synthesized using a DNA synthesizer, 381A (Applied Biosystems, Tokyo, Japan). Complementary strands were mixed in equal amounts, heated to 65°C, then slowly cooled to 37°C. The double-stranded oligonucleotides obtained were 5′-phosphorylated with T4 polynucleotide kinase then inserted in various combinations into the *Hin*dIII site in front of the putative upstream activation region-deficient *PHO3* in pKBN153. The identity of the inserted sequences was confirmed by DNA sequencing.

2.5. *Electrophoretic mobility shift assay*

Yeast nuclear extracts were prepared from strain X2180-1A grown under the indicated conditions as described by Schneider et al. [14]. DNA fragments were end-labeled with [α-^{32}P]dCTP with Klenow fragment and used for DNA-protein binding reactions as described by Company et al. [15] with some modifications. Gel shift experiments were carried out in a 20 μl reaction mixture containing 1 ng of a DNA probe (5,000 cpm), 10 μg of the nuclear extracts, 1 μg of non-specific competitor DNA (poly(dI-dC)), 4 mM Tris-HCl (pH 8.0), 40 mM NaCl, 4 mM $MgCl_2$, 1 mM dithiothreitol and 4% glycerol. For specific competition, 100 ng of non-labeled oligonucleotide was added. The mixture was incubated at 30°C for 30 min and loaded onto a 12% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed at −70°C.

2.6. *Acid phosphatase and protein assay*

Acid phosphatase activity with *p*-nitrophenyl phosphate as a substrate was determined directly using whole cells as previously described [16]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per min. Protein was determined using a protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

3. RESULTS AND DISCUSSION

3.1. *Deletion analysis of* PH03 *promoter DNA*

The *PH05* and *PH03* genes are clustered and located on chromosome II in *S. cerevisiae* (Fig. 2). There are only 350-bp nucleotides between the *PH05* mRNA end and the *PH03* transcription initiation site [17]. To determine the upstream boundary of sequences necessary for transcription of *PH03*, we first constructed two plasmids derived form pRS315 with pAP20. One contained both *PH05* and *PH03* genes (pKE) and the other included the *PH03* gene flanked with the 367-bp upstream region (pKB). These plasmids were transformed into the yeast strain, ND2-1C (*pho3 pho5*), and cells were cultured in medium in the presence or absence of thiamine, in which the expression of *PH05* gene was completely repressed. As shown in Fig. 3, both cells with pKE or pKB expressed the *PH03* gene to the same extent, indicating that the upstream region extending to the *Pst*I site of the *PH03* is adequate for full expression of the gene.

To determine the essential regulatory regions, we constructed a series of plasmids bearing various deletions in the 5′-noncoding region of the *PH03* gene and the expression of the *PH03* gene was examined as described above. The results are summarized in Fig. 3. The *PH03* genes with a promoter truncated at nucleotide position −234 and those with a longer promoter conferred about 10–30-fold more T-rAPase activity in thiamine-free medium than in thiamine-supplemented medium, although they caused a partial decrease of *PH03* expression. However, the promoter truncated at position −204 showed significantly lower T-rAPase activity and no differential synthesis of the enzyme in response to the thiamine concentration. Furthermore, the *PH03* fragment with internal deletion of the region from −247 to −215 barely showed the activity. These findings indicate that a sequence critical for *PH03* regulation in yeast is located in the 20-bp region between nucleotides −234 and −215.

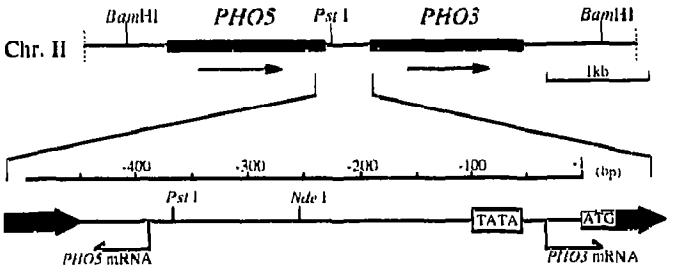

Fig. 2. Schematic diagram of the *PH03* promoter relative to the *PH05/PH03* locus.

Elimination of the upstream region from position −145 almost completely abolished the T-rAPase activity, whereas elimination of all the upstream region from position −127 resulted in a slight increase of the enzyme activity but no response to thiamine signals. These findings suggested that a 18-bp sequence from nucleotide −144 to −127 inhibits *PH03* expression (inhibitory region).

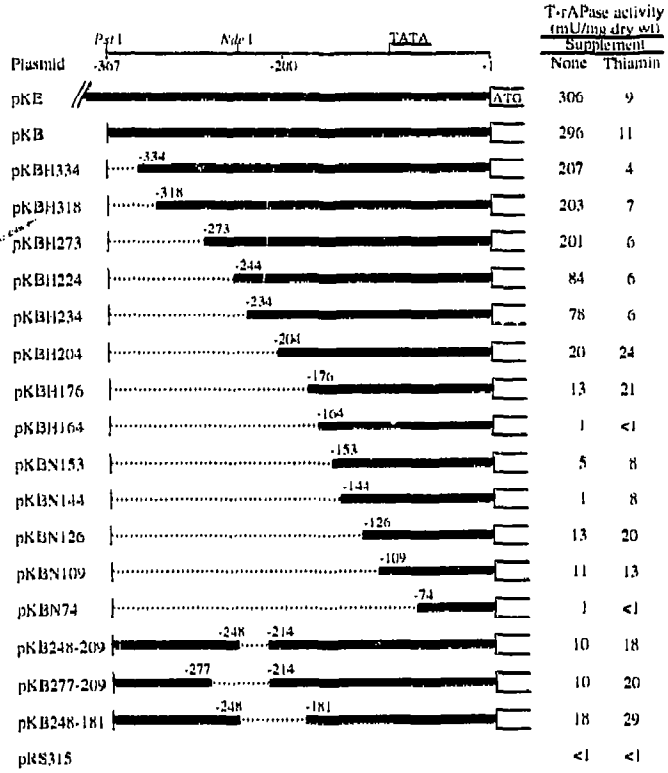

Fig. 3. Effect of deletions in the upstream region on *PH03* expression in the presence or absence of thiamine. The thick lines and open boxes represent the 5′-flanking region and the open reading frame of *PH03* DNAs, respectively. Numbers above the bars indicate nucleotide positions relative to the translation initiation codon. Each value is the mean of two experiments.

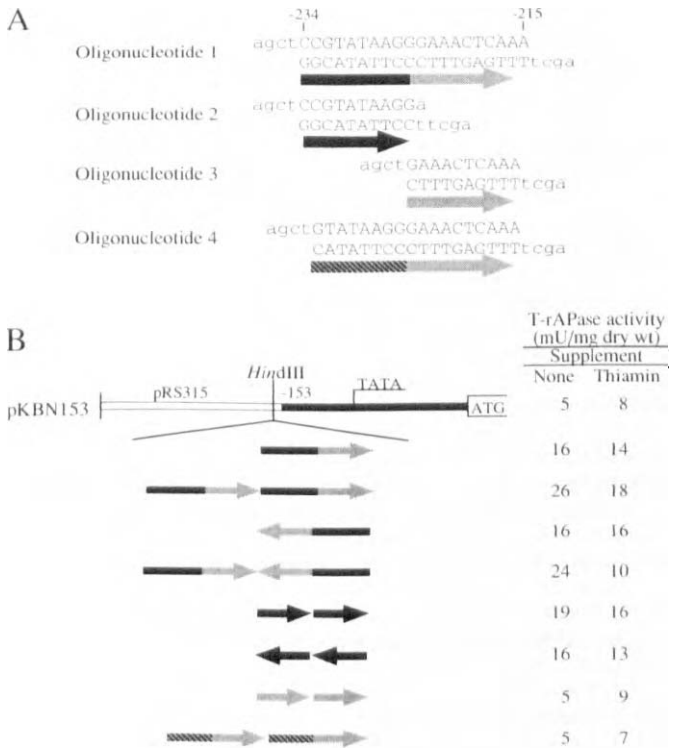

Fig. 4. Reconstitution of the upstream activation element from synthetic oligonucleotides. (A) Synthetic oligonucleotides used for reconstitution. The capital letters indicate synthetic nucleotides with the same sequences as authentic *PH03* promoter DNA, and the lowercase letters represent linker sequences. Each value is the mean of three experiments. (B) Expression of T-rAPase directed by the reconstituted upstream activation elements. The orientation of individual nucleotides in the constructs is indicated by the arrows.

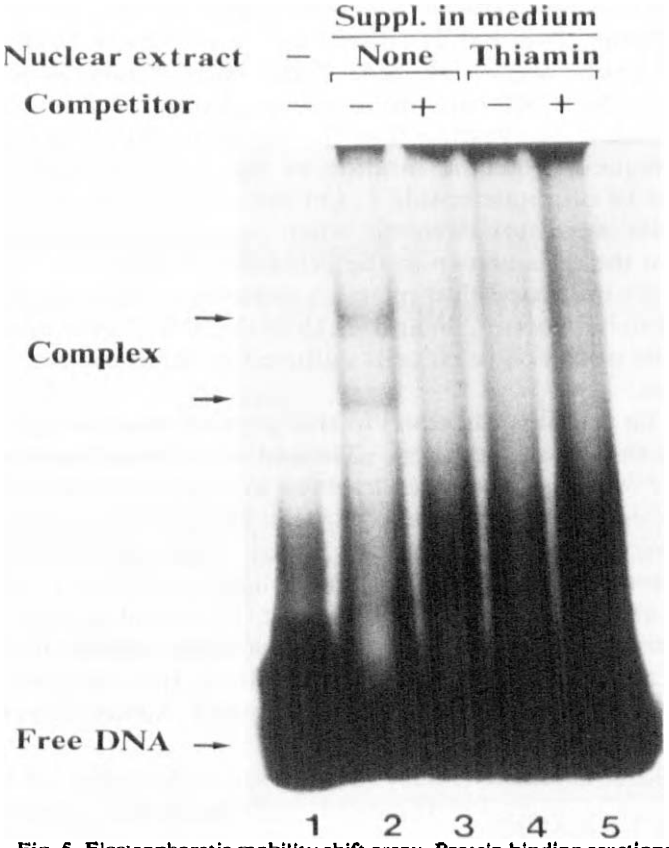

Fig. 5. Electrophoretic mobility shift assay. Protein binding reactions to ^{32}P-labeled oligonucleotide 1 were performed using yeast nuclear extracts from cells cultured in thiamine-free (lanes 2 and 3) or -supplemented medium (lanes 4 and 5) as described in Materials and Methods. The nuclear extracts were omitted from lane 1 and unlabeled nucleotides were added to the samples in lanes 3 and 5.

3.2. *Reconstruction of the upstream activation element*

Oligonucleotides were synthesized and used for the following reconstitution studies (Fig. 4). Oligonucleotide 1 covered the 20-bp region between nucleotides −234 and −215, which contained both sequences of oligonucleotides 2 (from −234 to −225) and 3 (from −226 to −215). Oligonucleotide 4 containing an 18-bp region excluded a 5′-flanking 2-bp nucleotide from oligonucleotide 1. All nucleotides were flanked by *Hin*dIII sites, and inserted, alone or in combination, into the *Hin*dIII site of pKBN153. These plasmids were introduced into strain ND2-1C, and the transformants were assayed for T-rAPase activities. Insertion of a single copy of oligonucleotide 1 in either orientation permitted a low but significant expression, which increased by connecting two copies of this oligonucleotide. In the latter, *PH03* expression was somewhat sensitive to thiamine. Two copies of oligonucleotide 2 also caused significant T-rAPase activity, but oligonucleotides 3 or 4 did not increase the expression.

These results suggested that the 20-bp region between −234 and −215 mediates both *PH03* gene expression and thiamine repression, although this region is not sufficient for full expression of the gene. In fact, when oligonucleotide 1 modified with the *Xho*I linker was inserted into the *Xho*I site in front of a UAS-deficient *CYC1-lacZ* fusion on a plasmid derived from pLG669-Z [18] (provided by S. Harashima, Osaka University), and when the resultant plasmid was transformed into a wild-type strain, the yeast cells expressed low levels of β-galactosidase in thiamine-free medium (data not shown). Since the deletions of the region −367 to −335 and the region −273 to −245 in the *PH03* promoter resulted in the decrease of the expression without effect for thiamine regulation (Fig. 3), these regions seem to be required for full expression of the gene together with the region between −234 and −215.

3.3. *Protein binding to oligonucleotide 1*

Since the region between −234 and −215 is likely to be responsible for repression by thiamine, we used the electrophoretic mobility shift assay to detect specific protein factors in nuclear extracts which can recognize this sequence. Oligonucleotide 1 was labeled with ^{32}P by filling in the two protruding ends. The resulting DNA being 28-bp in length, as a result of the fill-in reaction, was used as a DNA probe in the gel retardation reaction assay. As shown in Fig. 5, when the probe was mixed

with nuclear extracts from cells grown in the absence of thiamine, two bands migrating more slowly than the free probe were detected. These shift bands were removed by a 100-fold molar excess of unlabeled oligonucleotide 1, suggesting that the mobility shifts were due to sequence-specific binding of the yeast nuclear proteins to oligonucleotide 1. On the other hand, the shift bands were not detected when using nuclear extracts from the cells grown in the presence of thiamine. These results indicated that proteins capable of binding to the region between −234 and −215 of the *PH03* gene existed in the nuclei of yeast cells cultured in thiamine-free medium.

The findings obtained in this present study suggested that the region between −234 and −215 is indispensable for *PH03* expression and acts as an upstream activating region, which can interact with a regulatory protein(s). However, this region alone is not sufficient for *PH03* expression, because synthetic oligonucleotide 1 could not express the *PH03* gene to the full extent in vivo. To determine whether the sequences homologous to this region exist in other genes encoding the enzymes involved in thiamine metabolism in yeast, we are currently cloning these genes.

REFERENCES

[1] Toh-e, A., Kakimoto, S. and Oshima, Y. (1975) Mol. Gen. Genet. 143, 65–70.
[2] Schweingruber, M.E. (1987) in: Advances in Protein Phosphatases, vol. 4, (Merlevede, W. and Di Salvo, J., eds.), pp. 77–93, Leuven University Press, Leuven.
[3] Nishimura, H., Kawasaki, Y., Kaneko, Y., Nosaka, K. and Iwashima, A. (1992) FEBS Lett. 297, 155–158.
[4] Kawasaki, Y., Nosaka, K., Kaneko, Y., Nishimura, H. and Iwashima, A. (1990) J. Bacteriol. 172, 6145–6147.
[5] Nishimura, H., Kawasaki, Y., Nosaka, K., Kaneko, Y. and Iwashima, A. (1991) J. Bacteriol. 173, 2716–2719.
[6] Guarente, L. (1987) Annu. Rev. Genet. 21, 425–452.
[7] Struhl, K. (1989) Annu. Rev. Biochem. 58, 1051–1077.
[8] Tait-Kamradt, A.G., Turner, K.J., Kramer, R.A., Elliott, Q.D., Bostian, S.J., Thill, G.P., Rogers, D.T. and Bostian, K.A. (1986) Mol. Cell Biol. 6, 1855–1865.
[9] Sikorski, R.S. and Hieter, P. (1989) Genetics 122, 19–27.
[10] Wickerham, L.J. (1951) US Dept. Agric. Tech. Bull. 1029, 1–56.
[11] Ito, H., Fukuda, Y. Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[12] Sambrook, J. Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Schneider, R., Gander, I., Müller, U., Mertz, R. and Winnacker, E.L. (1986) Nucleic Acids Res. 14, 1303–1317.
[15] Company, M., Adler, C. and Errede, B. (1988) Mol. Cell Biol. 8, 2545–2554.
[16] Schweingruber, M.E., Fluri, R., Maundrell, K., Schweingruber, A.-M. and Dumermuth, E. (1986) J. Biol. Chem. 261, 15877–15882.
[17] Bajwa, W., Meyhack, B., Rudolph, H., Schweingruber, A.-M. and Hinnen, A. (1984) Nucleic Acids Res. 12, 7721–7739.
[18] Guarente, L. and Ptashne, M. (1981) Proc. Natl. Acad. Sci. USA 78, 2199–2203.