Volume 266, number 1,2, 78–82 FEBS 08514 June 1990

Disruption of the gene encoding subunit VI of yeast cytochrome bc_1 complex causes respiratory deficiency of cells with reduced cytochrome *c* levels

Chong H. Kim[1] and Richard S. Zitomer[2]

[1]*Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12181* and [2]*Department of Biological Sciences, State University of New York at Albany, Albany, NY 12222, USA*

Received 17 April 1990

A double mutant of *Saccharomyces cerevisiae*, in which *CYC1* gene is deleted and the chromosomal copy of the 17 kDa protein gene is disrupted, has been constructed. This mutant cannot grow on nonfermentable carbon sources, but normal growth can be restored by complementation of either mutation with a yeast vector containing either the wild-type 17 kDa protein gene or the *CYC1* gene. These results show that although the 17 kDa protein, subunit VI of yeast cytochrome bc_1 complex is dispensable for yeast mitochondrial respiration in cells with the wild-type levels of cytochrome *c*, the 17 kDa protein is essential for respiration when the level of cytochrome *c* is limited, indicating that is plays a role in electron transport. This glycerol$^-$ phenotype of the double mutant can serve as the basis for further genetic studies on the function of the 17 kDa protein in yeast mitochondria and may provide insight into the physiological function of the hinge protein, the counterpart of the yeast 17 kDa protein, in beef heart mitochondria.

Yeast 17 kDa protein; Cytochrome bc_1 complex; Glycerol$^-$ mutant; Respiration

1. INTRODUCTION

Ubiquinol cytochrome *c* oxidoreductase, the cytochrome bc_1 complex, is a mitochondrial respiratory enzyme complex common to eukaryotic energy transducing systems and some bacterial systems. This enzyme complex transfers electrons from ubiquinol to cytochrome *c*, and is coupled with proton translocation, thus generating ATP [1–4]. The cytochrome bc_1 complex isolated from beef heart mitochondria consists of 11 subunits [5]. Three of these subunits, cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein, have been well characterized with respect to their structure and function, but the roles of the remaining subunits have not been elucidated. One of these subunits, the hinge protein, which was originally identified in the beef heart bc_1 complex [6–10], has been implicated in the interaction of cytochrome c_1 with cytochrome *c*; it appears to regulate electron transfer between these two cytochromes [10,11]. Further studies on the function of this protein in mitochondria have been difficult, due to the inability to selectively modify or remove the hinge protein from the bc_1 complex. This has led us to search for another approach to elucidate the function of this protein, and we have initiated the studies with the cytochrome bc_1 complex of *Saccharomyces cerevisiae*, a genetically manipulable eukaryote in which respiration is not an essential function.

The yeast mitochondrial bc_1 complex consists of 8–10 subunits and is homologous to that of the beef heart mitochondria [1,2]. Recently, the sequence of the yeast gene encoding the 17 kDa protein, subunit VI of the bc_1 complex, has been reported [12], and the predicted amino acid sequence of this 17 kDa protein shows extensive homology (36%) to that of the beef heart hinge protein. The secondary structure of the 17 kDa protein also is predicted to be similar to that of the hinge protein of beef heart mitochondria. The sequence of the human hinge protein, which has been reported by Kagawa's laboratory [13], also showed 95% homology to that of the beef heart hinge protein.

Recently, Schoppink et al. [14–16] reported that the deletion of the gene encoding the 17 kDa protein had no significant effect on the cell growth, but resulted in reducing the ubiquinol cytochrome *c* reductase activity to 50% of that of the wild type, implying that this protein is not essential for activity and assembly of the complex. They also reported that extragenic mutations resulting in a respiratory deficient phenotype occurred at high frequencies in the 17 kDa protein deletion mutants. However, neither the significance nor the site of these secondary mutations has been determined.

Correspondence address: C.H. Kim, Department of Biology, RPI, Troy, NY 12181, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

In order to carry out the genetic studies on the function of the 17 kDa protein, it is necessary to establish a phenotype for the 17 kDa protein gene mutations. We report here that a double mutant, in which the 17 kDa protein is absent and the cellular cytochrome *c* levels are reduced to 5%, has a glycerol$^-$ phenotype and shows the respiratory capacity well below that of either single mutation or the additive effect of two single mutations. These results indicate that the 17 kDa protein might be necessary for mitochondrial respiration when the level of the cytochrome *c* becomes limiting.

2. EXPERIMENTAL

2.1. *Materials*

Restriction enzymes, Klenow fragment, and T4-DNA ligase were obtained from New England Biolabs, Boehringer Mannheim and US Biochemicals. Enzymatic reactions were carried out under the conditions recommended by the vendors. [α-^{32}P]dCTP was obtained from Amersham Co. The Gene Clean Kit was purchased from Bio101. All other reagents were the highest grade commercially available.

2.2. *Strains and transformation*

Saccharomyces cerevisiae strain HR2 and its derivative lacking the 17 kDa protein (17 kDa$^-$) were gifts of Leslie Grivell [15]. Strain aZ060 (*MAta, trp1-1, leu2-3,-112, his4-519, his3-11,-15, cyc1-1*) which is *iso*-1-cytochrome *c* deficient, was used in the construction of a mutant lacking both *iso*-1-cytochrome *c* and the 17 kDa protein. The yeast transformations were carried out as described [17]. The media for cell growth, YPD (2% glucose), YPG (3% glycerol), YPR (2% raffinose), and selective media (CM), have been described elsewhere [18]. Yeast cells were grown at 30°C with vigorous shaking. *Escherichia coli* JM109 [19] was used to propagate plasmid constructs. Bacterial transformations were carried out and cells were maintained as described [20].

2.3. *Plasmids*

The *TRP1* centromeric plasmids YCp*CYC1(2.4)* carrying the *CYC1* gene [21] and YCplac22 [22] were described previously. Plasmids p209 and 17 kDa-Leu-17 kDa were gifts from Leslie Grivell [15]. p209 consists of pFL1 into which a 4.9 kb yeast DNA fragment containing the *17 kDa* gene was inserted and 17 kDa-Leu-17 kDa contains a *LEU2* replacement of the 5′ noncoding and the beginning of the coding region of the *17 kDa* gene. This plasmid was digested with *Hin*dIII to carry out the one-step gene replacement [23] to generate a 17 kDa$^-$ mutant.

YCp*17 kDa** was constructed by ligating the 4.5 kb *Kpn*I-*Sal*I fragment containing the functional 17 kDa protein gene with the large *Kpn*I-*Sal*I fragment of YCplac22. The desired plasmid was identified by the restriction analysis. The large-scale preparations of plasmid DNA were carried out as described previously [24].

2.4. *Southern blot analysis*

Yeast DNA was prepared [25] and Southern analysis was carried out as described [26]. The radiolabeled DNA probe was generated by the random primer method [27].

2.5. *Preparation of mitochondria*

Mitochondria were prepared from cultures in exponential growth as described by Mason et al. [28].

* The gene for the 17 kDa protein is expressed in italic *17 kDa*, since this gene has not been named yet

2.6. *Enzymatic activity*

Succinate cytochrome *c* reductase activity was measured spectrophotometrically at room temperature by measuring the reduction of cytochrome *c* as previously reported [29].

2.7. *Oxygen consumption*

The rate of oxygen utilization was determined for cells grown to mid-log phase in YPR media using a polarographic method. 2 ml of culture was placed into a chamber mounted with a Clarke-type oxygen electrode, and the decrease in oxygen concentration over time was determined at 28°C.

3. RESULTS

3.1. *Construction of* cyc1-1,17 kDa$^-$ *mutant*

Schoppink et al. [14–16] reported that a deletion of the 17 kDa protein gene did not affect the growth rate of yeast cells on nonfermentable energy sources. We confirmed this result (see Table I) as well as their finding that a deletion of the *17 kDa* gene reduced the cytochrome *c* reductase activity to 40–50% of that of the wild type.

Since the hinge protein of the beef heart bc_1 complex is proposed to play a role in the interaction between the cytochromes bc_1 and *c*, we hypothesized that its yeast counterpart would function in a similar way so that the 17 kDa protein might be essential for respiration of cells containing limited amount of cytochrome *c*. *S. cerevisiae* contains two cytochrome *c* genes, the major gene *CYC1* encoding 95% of the cytochrome *c* in cells [30] and *CYC7* encoding the remaining 5% [31]. Deletion of the *CYC1* gene reduces the cytochrome *c* levels to 5% of that of the wild type, but only marginally affects the growth on glycerol, ethanol, or acetate, although such cells cannot grow on lactate [32].

The strain aZ060, which contains the *cyc1-1* allele, a deletion of the *CYC1* gene, was used to construct the double mutant through a one-step disruption of the *17 kDa* gene. Both glycerol$^+$ and glycerol$^-$ transformants were obtained, and a Southern analysis was performed with *Cla*I digested chromosomal DNA from both types of transformants to determine which transformant contained the fragments predicted from the disruption (Fig. 1A). As shown in Fig. 1B, the wild-type (for the *17 kDa* gene) yeast strain, aZ060 and glycerol$^+$ transformants (lane 3) showed a hybridization band of 2.8 kb characteristic of the wild type *17 kDa* gene. The glycerol$^-$ transformants (lanes 1 and 2) showed the 3.5 kb band expected from the integration of the *LEU2* disruption at the *17 kDa* locus (see Fig. 1A for the predicted fragments). One *cyc1-1,17 kDa*$^-$ double mutant, designated aZ060-17, was picked for further investigation.

3.2. *Complementation of the double mutant aZ060-17 with plasmids YCp*17 kDa *or YCp*CYC1(2.4)

To confirm if the glycerol$^-$ phenotype was indeed due to the deletion of the *17 kDa* gene, aZ060-17 cells were transformed with centromeric plasmids contain-

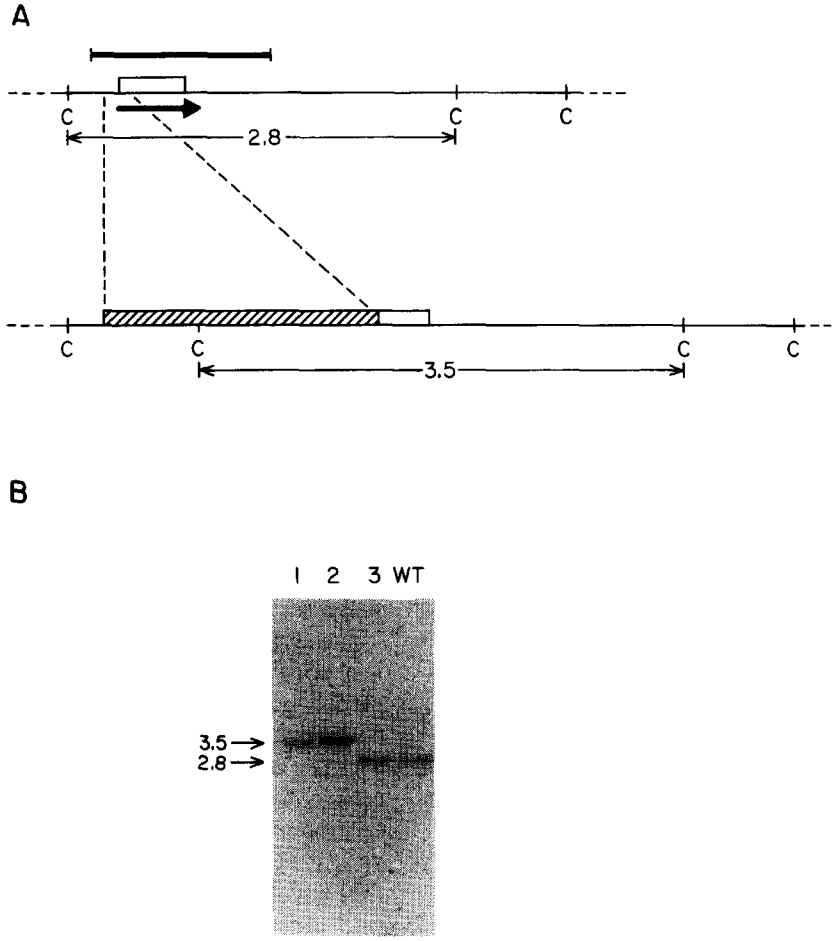

Fig. 1. Southern analysis. (A) The predicted physical maps of the yeast genome at the wild-type 17 kDa locus and at the *LEU2*-17 kDa gene disruption are shown. C represents the restriction site for *Cla*I. The 17 kDa protein gene locus is marked in a rectangular box and *LEU2* gene is shown in a shaded box. A solid bar above the 17 kDa protein gene indicates an *Acc*I fragment (1.3 kb) which was used as a DNA probe. (B) Genomic DNA isolated from leu$^+$ transformants of aZ060, glycerol$^-$ phenotype (lanes 1, 2), glycerol$^+$ phenotype (lane 3) and aZ060 wild-type (WT), were digested with *Cla*I and transferred to a nylon membrane. The autoradiogram was obtained by hybridization of the bound DNA to the ^{32}P labeled *Acc*I fragment. DNA lengths (kb) were determined from a Lambda *Bst*EII digest visualized with ethidium bromide in the original gel.

ing either the *17 kDa* gene (YCp*17 kDa)*) or the *CYC1* gene (YCp*CYC1(2.4)*) to complement the respective deletions. If the glycerol$^-$ phenotype resulted solely from the combined deletions and not from a third site mutation, then the restoration of either gene should result in a glycerol$^+$ phenotype. Six independent trp$^+$ transformants for each plasmid were tested for the growth on glycerol. Sample results are shown in Fig. 2; the introduction of either the wild-type *CYC1* or *17 kDa* gene into aZ060-17 cells restored growth on glycerol, while the transformation of cells with the yeast vectors without either gene did not restore respiratory capacity.

In order to quantitatively compare these glycerol phenotypes, we have monitored the growth rates for 48 h of HR2 wild-type and the *17 kDa*$^-$ mutant, and aZ060, aZ060-17 and aZ060-17 transformed with either YCp*CYC1(2.4)* or YCp*17 kDa*, in liquid glycerol media. The doubling times are summarized in Table I. Deletion of either the *CYC1* or the *17 kDa* gene had only a marginal effect on the growth rate of cells in glycerol, but the double mutation caused a three-fold increase in the doubling time and this effect is more than the additive effects by two single mutations.

To confirm that this reduced growth rate reflected a reduced oxygen consumption, we measured the oxygen consumption rate in exponentially grown cells. Oxygen consumption in the double mutant was about two times slower than that of cells carrying the *CYC1* deletion and three times slower than that of cells carrying the *17 kDa* disruption (Table II). These results again support the conclusion that the slow growth rate of the double mutant is due to the reduced respiratory capacity of cells.

3.3. *Succinate cytochrome* c *reductase activity of isolated mitochondria*

As stated above, we have confirmed the difference in

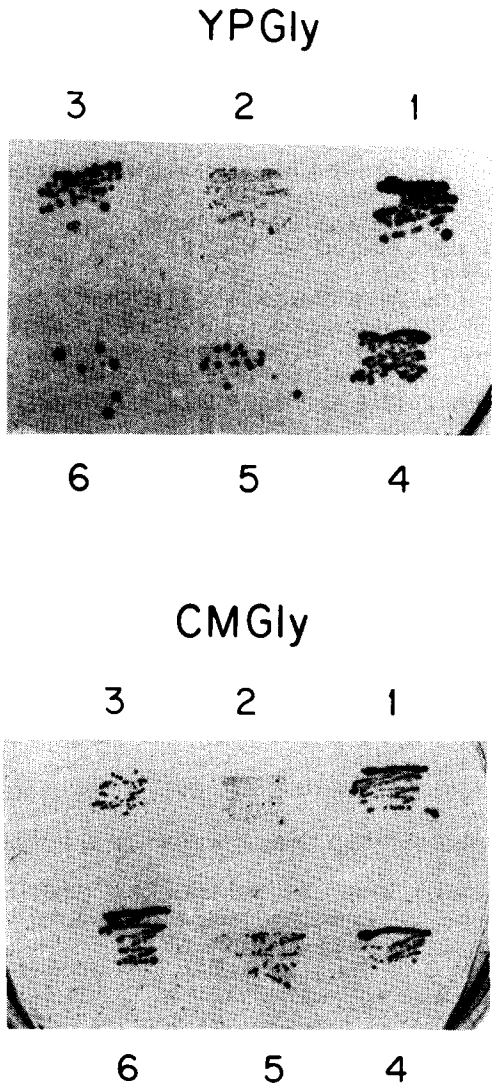

Fig. 2. Comparison of growth or respiratory sufficiency of aZ060 wild-type (1), aZ060-17 (2) and aZ060-17 transformants with plasmids carrying a *CYC1* (3 and 4) or 17 kDa (5 and 6) gene. Cells were plated on YPGly (3% glycerol) and CMGly (3% glycerol) media.

Table I

Doubling time of yeast strains cultured in liquid media containing a nonfermentable carbon source (YPGly)

Strain	Genotype	Plasmid	Doubling time (h)
HR2	*CYC1,17 kDa*$^+$	–	5.0
	CYC1,17 kDa$^-$	–	6.5
aZ060	*cyc1-1,17 kDa*$^+$	–	6.5
aZ060-17	*cyc1-1,17 kDa*$^-$	–	17.5
	cyc1-1,17 kDa$^-$	YCp*CYC1(2.4)*	5.5
	cyc1-1,17 kDa$^-$	YCp*17 kDa*	6.5

Culture temperature was 30°C

Table II

The oxygen consumption rate of yeast cells grown to mid-log phase in liquid raffinose media (YPR)

Strain	Plasmid	ΔO_2/min (%)[a]
aZ060	–	11.0
aZ060-17	–	3.7
aZ060-17	YCp*CYC1(2.4)*	10.5
aZ060-17	YCp*17 kDa*	8.0

[a] Expressed as the decrease of oxygen concentration (%) per minute per cell suspension of A_{550} = 0.5. ΔO_2 is average of six measurements for three different cultures for each strain. Cells were grown at 30°C and oxygen measurement was done at 28°C

Table III

The cytochrome *c* reductase activity in mitochondria from the strains, HR2 (wild-type, *17 kDa*$^-$ mutant) and aZ060 (*17 kDa*$^-$ mutant and its transformant with YCp*17 kDa*)

Strain	Plasmid	Spec. act.[a]	% Wild-type
HR2, wild-type	–	0.28	100
HR2, *17 kDa*$^-$	–	0.16	57
aZ060-17	–	0.09	32
aZ060-17	YCp*17 kDa*	0.197	70

[a] Expressed in μmol of cytochrome *c* reduced per min per mg protein of mitochondria

the cytochrome *c* reductase activity between the wild-type and the *17 kDa*$^-$ mutant of HR2 ([14–16], see Table III), using the mitochondria prepared from the respective cell cultures. The measurement of succinate cytochrome *c* reductase activity of the mutant, aZ060-17 and aZ060-17 containing the YCp*17 kDa* plasmid showed that the lack of a 17 kDa protein resulted in a 55% loss of the enzyme activity of the bc_1 complex compared to that of mitochondria prepared from cells carrying the wild-type gene. Since these assays were carried out in the presence of excess exogenous cytochrome *c*, the results demonstrated that the cytochrome bc_1 complex in the double mutant was functionally identical to that in the single *17 kDa*$^-$ mutant. Thus, in the double mutant, electron transfer from the bc_1 complex to cytochrome *c* becomes the rate-limiting step in respiration, and the 17 kDa protein presumably plays a role in this process.

4. DISCUSSION

The aim of the present study was to define conditions under which the 17 kDa protein is essential for respiration. Such conditions would allow a genetic analysis of the structure and function of this protein in the cytochrome bc_1 complex. The results presented here clearly show that, when cytochrome *c* levels are reduced, the 17 kDa protein becomes essential for reasonable growth on glycerol, thus satisfying the requirements for

genetic selections. In addition, the finding that efficient electron transport becomes dependent on the 17 kDa protein when cytochrome *c* is limiting, supports the current hypothesis for the role of 17 kDa protein in the interaction of the bc_1 complex with cytochrome *c*. Reconstitution and kinetic studies with the beef heart bc_1 complex have suggested such a role for the hinge protein, homologous to the 17 kDa protein.

Schoppink et al. [34] recently reported that the absence of the 17 kDa protein resulted in a decrease in the rate of reduction of both horse heart and yeast cytochromes *c* by the cytochrome bc_1 complex under high ionic strength (>33 mM for horse heart cytochrome *c* and >225 mM for yeast cytochrome *c*). They proposed that the 17 kDa protein is responsible for the association of basic ferricytochrome *c* with the bc_1 complex and this binding becomes rate-limiting at high ionic strength in the absence of the 17 kDa protein. These results are consistent with our findings that the 17 kDa protein plays a role in the interaction of the bc_1 complex with the cytochrome *c* and thus becomes essential for cell respiration when the cytochrome *c* levels are limiting.

Although it has been reported that certain mutations in the 17 kDa protein might affect the assembly of the bc_1 complex or cytochrome *c* oxidase [33], the disruption of the gene used in our studies and those of others [15,34] had no effect on the assembly of the complexes; the intact bc_1 complex and functional mitochondria could be isolated from cells carrying such disruption. Further genetic investigation of the function of the 17 kDa protein would clarify this and such investigations are currently conducted in our laboratory.

Acknowledgements: This work was supported by grants from the American Heart Association and National Institutes of Health. We are grateful to Drs L. Grivell and J.A. Berden for their kind gifts of HR2 (the wild type and 17 kDa$^-$ mutant), plasmids p209 and 17 kDa-LEU-17 kDa. We thank Dr C. Lowry for his valuable suggestions and discussion.

REFERENCES

[1] Katan, M.B., Pool, L. and Groot, G.S.P. (1976) Eur. J. Biochem. 65, 95–105.
[2] Siedow, J.N., Power, S., De La Rosa, F.F. and Palmer, G. (1978) J. Biol. Chem. 253, 2392–2399.
[3] Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1105–1169.
[4] Yang, X. and Trumpower, B.L. (1986) J. Biol. Chem. 261, 12282–12289.
[5] Schagger, H., Link, Th.A., Engel, W.D. and Von Jagow, B. (1986) Methods Enzymol. 126, 224–237.
[6] Kim, C.H. and King, T.E. (1981) Biochem. Biophys. Res. Commun. 101, 29–36.
[7] Wakabayashi, S., Takeda, H., Matsubara, H., Kim, C.H. and King, T.E. (1982) J. Biochem. 91, 2077–2085.
[8] Kim, C.H. and King, T.E. (1983) J. Biol. Chem. 258, 13543–13551.
[9] Kim, C.H. (1987) in: Advances in Membrane Biochemistry and Bioenergetics (Kim, C.H., Tedeschi, H., Diwan, J.J. and Salerno, J.C. eds) pp. 167–178, Plenum, New York.
[10] Kim, C.H., Balny, C. and King, T.E. (1987) J. Biol. Chem. 212, 8103–8108.
[11] Kim, C.H., Balny, C. and King, T.E. (1987) in: Cytochromes; Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L. eds) pp. 415–422, Plenum, New York.
[12] Van Loon, A.P.G.M., De Groot, R.J., De Haan, M., Dekker, A. and Grivell, L.A. (1984) EMBO J. 3, 1039–1043.
[13] Ohta, S., Goto, K., Arai, H. and Kagawa, Y. (1987) FEBS Lett. 226, 171–175.
[14] Schoppink, P.J., Grivell, L.A. and Berden, J.A. (1987) in: Advances in Membrane Biochemistry and Bioenergetics (Kim, C.H., Tedeschi, H., Diwan, J.J. and Salerno, J.C. eds) pp. 129–139, Plenum, New York.
[15] Schoppink, P.J., Hemrika, W., Reyner, J.M., Grivell, L.A. and Berden, J.A. (1988) Eur. J. Biochem. 173, 115–122.
[16] Berden, J.A., Van Hoek, A.N., De Vries, S. and Schoppink, P.J. (1987) in: Cytochromes; Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L. eds) pp. 523–531, Plenum, New York.
[17] Klebe, R.J., Harris, J.V., Sharp, Z.D. and Douglas, M.G. (1983) Gene 25, 333–341.
[18] Zitomer, R.S. and Hall, B.D. (1976) J. Biol. Chem. 251, 6320–6326.
[19] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 3, 103–119.
[20] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[21] Lowry, C.V., Weiss, J.L., Walthall, D.A. and Zitomer, R.S. (1983) Proc. Natl. Acad. Sci. USA 80, 151–155.
[22] Gietz, R.D. and Sugino, A. (1988) Gene 74, 527–534.
[23] Rothstein, R.J. (1983) Methods Enzymol. 101, 202–211.
[24] Elwell, L.P., De Graaff, J., Selbert, D. and Falkow, S. (1975) Infect. Immun. 12, 404–410.
[25] Stuhl, K., Stinchcomb, D.T., Scherer, S. and Davis, R.W. (1979) Proc. Natl. Acad. Sci. USA 76, 1035–1039.
[26] Southern, E.M. (1975) J. Mol. Biol. 98, 503–517.
[27] Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 132, 6–13 (Addendum 137, 266–267).
[28] Mason, T.L., Poyton, R.O., Wharton, D.C. and Schatz, G. (1973) J. Biol. Chem. 248, 1346–1354.
[29] King, T.E. (1967) Methods Enzymol. 10, 202–208.
[30] Sherman, F., Stewart, J.W., Margoliash, E., Parker, J. and Campbell, W. (1966) Proc. Natl. Acad. Sci. USA 55, 1498–1504.
[31] Downie, J.A., Stewart, J.W., Brockman, N., Schweingruber, A. and Sherman, F. (1977) J. Biol. Chem. 113, 369–384.
[32] Sherman, F., Stewart, J.W., Jackson, M., Gilmore, R.A. and Parker, J.H. (1974) Genetics 77, 255–284.
[33] Schmitt, M.E. and Trumpower, B. (1987) in: Cytochromes; Molecular Biology and Bioenergetics (Papa, S., Chance, B. and Ernster, L. eds) pp. 177–187, Plenum, New York.
[34] Schoppink, P.J., Hemrika, W. and Berden, J.A. (1989) Biochim. Biophys. Acta 974, 192–201.