The N-terminal, dehydrogenase/cyclohydrolase domain of yeast cytoplasmic trifunctional C1-tetrahydrofolate synthase requires the C-terminal, synthetase domain for the catalytic activity in vitro

Jae Mahn Song, Jesse C. Rabinowitz*

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

Received 4 October 1995; revised version received 25 October 1995

Abstract **The yeast *ADE3*(1-333) gene which encodes a truncated protein containing the N-terminal 5,10-methylene-tetrahydrofolate (THF) dehydrogenase (D)/5,10-methenyl-THF cyclohydrolase (C) domain of cytoplasmic trifunctional C1-THF synthase is able to complement all the phenotypes associated with *ade3* mutations in vivo. However, expression of the *ADE3*(1-333) gene in an *ade3* strain does not retain any D activity in vitro. Expression in a yeast *ade3* strain of the *ADE3*(1-333) fused to the *Escherichia coli lacZ* gene or to the yeast *SER2* gene allows detection of D and C activities in vitro. These results indicate that the N-terminal D/C domain of C1-THF synthase requires the C-terminal 10-formyl-THF synthetase domain for stable catalytic activity in vitro.**

Key words: Yeast; Gene fusion; *ADE3*; C1-tetrahydrofolate

1. Introduction

The one-carbon derivatives of tetrahydrofolate (THF) serve as doners of one-carbon units in a variety of biosynthetic reactions involving purine bases (inosinic acid), pyrimidines (thymine), amino acids (methionine, serine, glycine), vitamins (pantothenic acid), and the initiation of protein synthesis in bacteria and organelles. The principal source of the one-carbon metabolite is 5,10-methylene-THF normally derived from glucose via glycine [1], although it is also formed in a non-enzymic reaction by condensation of formaldehyde with THF.

An enzymatic activity resulting in the formation of 10-formyl-THF in the presence of formate, THF and Mg-ATP was found in almost all cells examined, including those of bacteria, plants, yeasts and animals [1]. The only exception reported to date, however, is the absence of the enzyme from *Escherichia coli* and other enteric bacteria [2,3]. However, yeast [4] and vertebrate species [1] unlike prokaryotes, contain enzymes that are trifunctional. They possess in addition to the 10-formyl-THF synthetase (S) (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (C) (EC 3.5.4.9) and 5,10-methylene-THF dehydrogenase (D) (EC 1.5.1.5) activities. The trifunctional enzyme was named C1-THF synthase. D catalyzes the oxidation of 5,10-methylene-THF to 5,10-methenyl-THF and C catalyzes the hydrolysis of 5,10-methenyl-THF to 10-formyl-THF. D and C occur as monofunctional or bifunctional proteins in prokaryotes. The monofunctional forms are found in *Clostridium cylindrosporum* [5] and the bifunctional form is found in *C. thermoaceticum* and *E. coli* [6,7].

The genes yielding the trifunctional enzymes have been cloned and sequenced from yeast cytoplasm (*ADE3*) [8] and mitochondria (*MIS1*) [9], and several other eukaryotic sources [10,11]. Comparison of the primary structures of the C1-THF synthases, deduced from the nucleotide sequences, reveals extensive homology among the proteins. The C1-THF synthases are composed of two identical polypeptides. Proteolysis experiments with mammalian C1-THF synthases have shown that the protein can be separated into two functional domains: the N-terminal domain containing the D/C activities and the C-terminal domain containing the S activity, respectively [12,13]. Hum and MacKenzie have demonstrated that both domains of human C1-THF synthase form active enzymes and fold independently in *E. coli* [14]. In contrast, treatment of the yeast cytoplasmic C1-THF synthase with trypsin resulted in the decay of the D/C activities of the N-terminal domain, but the S activity of the C-terminal domain was retained [4].

In this report, we have examined the C1-THF synthase activities in an *ade3* deletion strain transformed with a plasmid carrying the *ADE3*(1-333) gene [15] (B and C, Fig. 1) coding for a truncated protein retaining the D/C domain but not the S domain of C1-THF synthase. Additional constructions were made in which the *E. coli lacZ* and the yeast *SER2* gene replaced the S portion of the *ADE3* gene by fusing the *E. coli lacZ* gene to *ADE3*(1-333) and the *SER2* gene to *ADE3*(1-666), respectively (D and E, Fig. 1).

2. Materials and methods

2.1. Strains and media

The *Saccharomyces cerevisiae* strains used were GT48 (*MATa ade3*-130 *ser1*-171 *ura3*-52), S40 (*MATa ade3*-130 *mis1*-5 *ser1*-171 *ura3*-52), and CBY4 (*MATa ade3*-65 *ser1*-171 *ura3*-52 *trp1 leu2 his3 his4*). The *ade3*-130 and *ade3*-65 alleles are *ade3* deletion and D-deficient mutations, respectively [16,17]. The *mis1*-5 allele is a *mis1* disruption mutation with *ura3*$^-$. Strain S40 carrying the *mis1*-5 was isolated as a *ura3*$^-$ strain able to grow on 5-fluoroorotic acid medium from the *mis1*::*URA3* disruption mutant, which was constructed in strain GT48 through the gene replacement with plasmid pKS23 linearized with *Eco*RI and *Sal*I [18]. SD medium is 0.17% yeast nitrogen base/0.5% ammonium sulfate/2% glucose and was supplemented with the following nutrients where indicated (final concentration in mg/l): adenine, 20; histidine, 20; serine, 400; uracil, 20; leucine, 30; tryptophan, 20; glycine, 20; and formate, 1,000. Yeast transformations were performed by treatment with lithium acetate [19]. *E. coli* DH5α was used to propagate plasmids.

*Corresponding author. Fax: (1) (510) 643-5035.
E-mail: panisse@mendel.berkeley.edu

Abbreviations: THF, tetrahydrofolate; S, 10-formyl-THF synthetase; C, 5,10-methenyl-THF cyclohydrolase; D, 5,10-methylene-THF dehydrogenase.

SSDI 0014-5793(95)01288-5

2.2. Plasmids

Plasmids pJS8A and pJS8C are derivatives of centrometric plasmid YCp50 and episomal multicopy plasmid YEp24, respectively, and both carry the *URA3* and *ADE3* genes [15]. Plasmid pJS8A-A, containing the *URA3* and *ADE3*(1-333) genes, is a derivative of YCp50 [15]. It was used to construct plasmid pBJL1 carrying the *URA3* and the *ADE3*(1-333)–*lacZ* fusion gene. The *ADE3*(1-333)–*lacZ* fusion gene was created by inserting a 6.8-kb *Bam*HI fragment containing *E. coli lacZ* gene of pMC931 [20] into the *Hin*dIII site of the modified pJS8A-A, through the use of synthetic linkers and blunt-end ligation. This fusion gene consisted of the *ADE3*(1-333) fused to the *lacZ* gene with its first 7 codons removed (Fig. 1D). Multicopy plasmid pJS54 carrying the *ADE3*(1-666)–*SER2* fusion gene was constructed by removing the 4.1-kb *Kpn*I fragment from the multicopy plasmid pJS8C to yield the *ADE3*(1-666) fused to the *SER2* gene (Song, J.M., Cheung, E. and Rabinowitz, J.C., GenBank/EMBL database with the accession number U36473). The removal of the 4.1-kb *Kpn*I fragment including the last 281 codons of the *ADE3* and the first 40 codons of the *SER2* from pJS8C created the *ADE3*(1-666)–*SER2* fusion gene (Fig. 1E). The multicopy plasmid pJS50 carrying the *ADE3*(1-333) gene was constructed by removing the 1.5-kb *Hin*dIII fragment in the S domain of the *ADE3* gene of multicopy plasmid YEpADE3 [8]. Plasmid pKS23 is a derivative of pUC4 and carries a *mis1* :: *URA3* allele created by replacing the 56-bp *Xho*I–*Sph*I fragment at the 5′-end of *MIS1*-coding sequence with a 1.1-kb fragment containing the *URA3* gene [9].

2.3. Determination of growth rate

Cultures were grown in 125 ml flasks at 30°C in a shaking water bath. Yeast cells carrying the *URA3*-bearing plasmids were grown in SD supplemented with adenine, histidine and serine. Growth rates of all strains were determined in SD medium supplemented with the indicated nutrients. Cells grown to an OD_{600} of 1 were harvested and washed in SD. Cells were then resuspended in SD and used to inoculate SD supplemented with the appropriate nutrients. Growth was monitored by measuring the OD_{600}.

2.4. Enzyme assays

Yeast cells carrying plasmids were grown in SD supplemented with appropriate nutrients to an OD_{600} of 1, and harvested by centrifugation at 4°C. The cell pellets were resuspended in 2 volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM 2-mercaptoethanol, and were disrupted with glass beads. Protease inhibitors, when used alone or in combination, were added to the buffer just before disrupting the cells with glass beads. PMSF, phenanthroline and benzamidine were added to final concentrations of 1 mM, 5 μM and 10 μM, respectively. Pepstatin A, leupeptin, chymostatin, aprotinin and antipain were added to a final concentration of 0.5 μg/ml. The lysates were then centrifuged at 25,000 × *g* for 30 min. The supernatant fractions were used for enzyme and protein assays. The S and D activities were assayed as previously described [16]. The activities are expressed in milli-units (nmols of 5,10-methenyl-THF formed or hydrolyzed/min) based on $\varepsilon = 24{,}900\ M^{-1}\cdot cm^{-1}$ for 5,10-methenyl-THF. The C activity was measured at room temperature as described [21]. 1 milli-unit is equivalent to the formation of 1 nmol of product/min. Quantitative β-galactosidase (β-Gal) assays were carried out as described [22]. The β-Gal activity is expressed in units (nmols of 2-Nitrophenyl-β-D-galactopyranosid hydrolyzed/min). Protein concentrations were determined by the dye-binding assay of Bradford [23] with bovine serum albumin as a standard.

3. Results and discussion

3.1. Effect of expression of the ADE3*(1-333) gene in* ade3 *strains*

It has previously been shown that the plasmid-born N-termi-

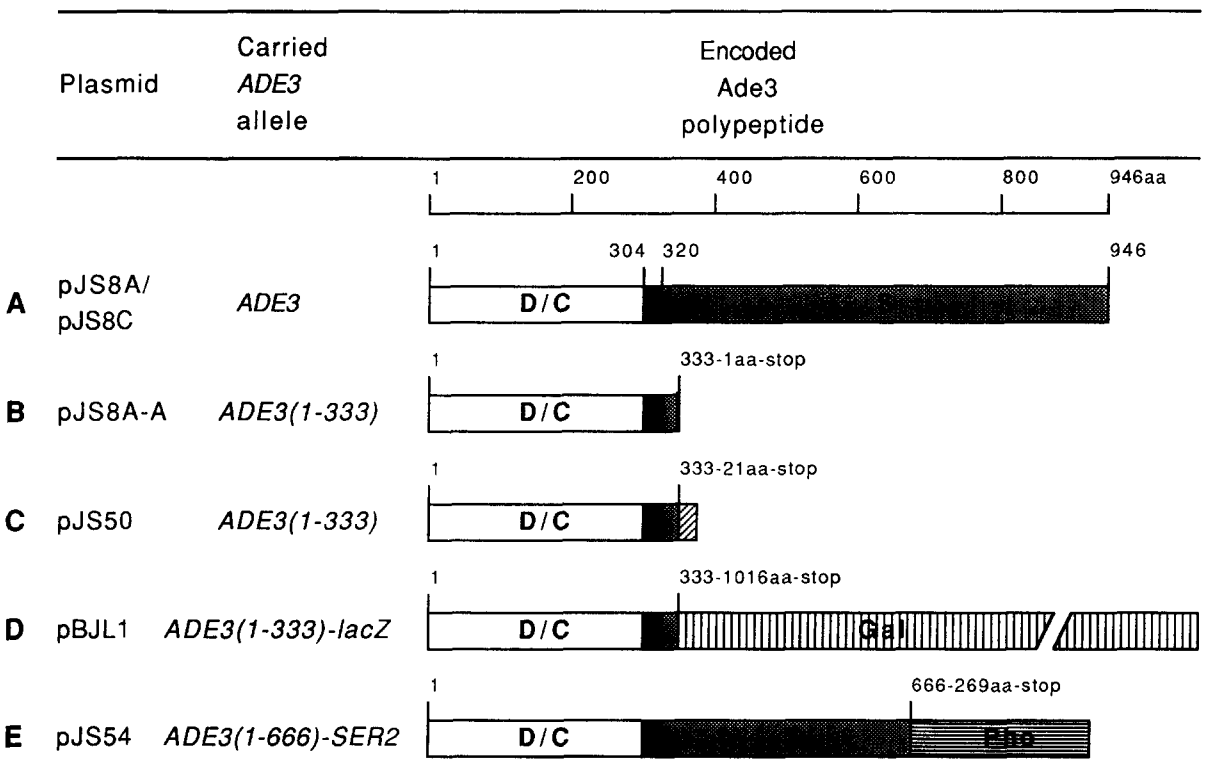

Fig. 1. Ade3 polypeptides encoded by *ADE3* alleles. Based on the homology to the amino acid sequences of procaryotic counterparts, Ade3 polypeptide is composed of a 304 amino acid N-terminal D/C domain (open box), a 627 amino acid C-terminal S domain (stippled box), and a 15 amino acid interdomain (black box). The C-terminal regions of truncated proteins are deduced from the nucleotide sequences. The number of the created amino acid insertions (hatched box) is indicated before 'stop' for the termination codon. The fusion proteins contain β-galactosidase (Gal) with the first 7 amino acids removed (vertically hatched box) and phosphoserine phosphatase (Pho) with the removed first 40 amino acids (horizontally hatched box).

Table 1
Phenotypes of *ade3 mis1* strain S40 with plasmids carrying different *ADE3* alleles

Plasmid	Carried *ADE3* allele	Growth rate (doubling time)		Enzyme activity[a]			
		+ Adenine	− Adenine	D	C	S	β-Gal
		h		mU/mg			U/mg
YCp50	None (vector)	2.2	−[b]	0.2	0	0	0
pJS8A	*ADE3*	2.2	2.2	18	42	151	0
pJS8A-A	*ADE3*(1-333)	2.1	2.1	0	3.8	0	0
pBJL1	*ADE3*(1-333)–*lacZ*	2.2	2.2	8.2	14.5	0	493

[a] The value below 1 mU/mg for the D activity could be an artifact of the assay method, since the boiled lysates sometimes show values below 1 mU/mg.
[b] −, No sign of growth by 3 days.

nal portion of the *ADE3* gene, *ADE3*(1-333), coding for a truncated protein retaining the D/C domain but not the S domain, is sufficient for complementation of the adenine requirement associated with an amber mutation *ade3*-26 [15]. We have now determined the effect of expression of the *ADE3*(1-333) gene on the phenotypes associated with *ade3* mutations in other *ade3* strains. First, its effect on the adenine requirement associated with the *ade3* mutation was examined in the *ade3* deletion strain GT48. The GT48 strain transformed with plasmid pJS8A-A (Fig. 1B) carrying the *ADE3*(1-333) gene does not require adenine for growth. Second, an alternative phenotypic assay for catalytic function of the *ADE3* C1-THF synthase not related to the adenine requirement is based on the inability of *ade3 ser1* mutants to satisfy their serine requirement by utilizing glycine and formate to synthesize serine [24]. To test this catalytic ability, plasmid pJS8A-A was transformed into *ade3*-65 *ser1* strain CBY4. The *ade3*-65 mutant exhibited wild-type levels of S and C activities but little D activity above that derived from the mitochondrial C1-THF synthase and little growth on +Glycine/formate [17]. The CBY4 transformant with the plasmid pJS8A-A which is able to complement the adenine requirement in *ade3* deletion strain GT48, grows well on +Glycine/formate like the one with plasmid pJS8A carrying the *ADE3* gene. These results indicate that the *ADE3*(1-333) gene encoding a truncated protein containing the N-terminal D/C domain is able to complement all the phenotypes associated with *ade3* mutations in vivo. Then, we analyzed crude extracts of the GT48 strain transformed with the plasmid pJS8A-A for the enzymatic activities. The specific activity of D in the transformant with the plasmid pJS8A-A was 6.4 mU/mg, while that of the transformants with the plasmids YCp50 and pJS8A were 5.2 and 26.1 mU/mg, respectively. These results indicate that the transformant with the plasmid pJS8A-A showed little D activity above that derived from the mitochondrial C1-THF synthase. In order to determine the enzymatic activities of cytoplasmic C1-THF synthase, we have disrupted the *MIS1* gene in the *ade3* deletion strain GT48. The S40 strain was then transformed with the plasmid pJS8A-A. The transformant does not require adenine for growth. Enzyme assays show that it has no D activity but a little C activity (Table 1).

One explanation for the lack of demonstrable D activity of the S40 strain transformed with the plasmid pJS8A-A is that its D activity is barely detected in vitro, since its C activity is also lower than the one of the S40 strain transformed with the plasmid pJS8A. To investigate this possibility, a multicopy plasmid pJS50 carrying the *ADE3*(1-333) gene was constructed. Results of enzyme assays in crude extracts prepared from the S40 strain transformed with the plasmid pJS50 are shown in Table 2. The transformant with the multicopy plasmid pJS50 exhibits the 11-fold higher C activity than the one with the low copy plasmid pJS8A-A. However, it still does not have any D activity (Tables 1 and 2).

Another possibility for the inability to detect D activity is that the D activity of the transformant is destroyed by proteases during the preparation of crude extracts, since we do not use any protease inhibitor in the preparation of crude extracts for C1-THF synthase. We therefore tested eight commonly used protease inhibitors alone or in combination. However, none of the treated extracts showed much D activity. We also have been unable to detect any D activity in extracts prepared at 0°C and assayed as quickly as possible (~30 min after cell breakage).

In summary, the N-terminal D/C domain of C1-THF synthase encoded by the *ADE3*(1-333) gene is active in vivo by complementing all the phenotypes associated with *ade3* mutations. However, extracts of these cells have no detectable D activity in vitro. Addition of protease inhibitors during preparation of cell extracts does not have any effect on our inability to detect D activity in extracts. These results suggest that the D/C activities of the N-terminal domain of C1-THF synthase may be affected by the C-terminal domain in vitro.

3.2. *Effect of expression of fusion genes in* ade3 *strain*

To examine the effect of the C-terminal domain of the C1-THF synthase on the D/C activity of its N-terminal domain, we have constructed the *ADE3*(1-333) encoding a truncated protein containing the N-terminal D/C domain of C1-THF synthase fused to *E. coli lacZ* encoding β-galactosidase. The constructed low copy plasmid pBJL1 carrying the fusion gene *ADE3*(1-333)–*lacZ* was then transformed into *ade3 mis1* strain S40. The transformant does not require adenine for growth, suggesting that the fusion gene is active in vivo. Results of enzyme assay in crude extracts prepared from the transformant are shown in Table 1. Indeed, the transformant with the plasmid pBJL1 carrying the *ADE3*(1-333)–*lacZ* fusion gene re-

Table 2
Enzymatic activities of overexpressed Ade3 proteins in *ade3 mis1* strain S40

Plasmid	Carried *ADE3* allele	Enzymatic activity[a] (mU/mg)		
		D	C	S
YEp24	Vector	0.1	0	0
pJS8C	*ADE3*	184	454	1686
pJS50	*ADE3*(1-333)	0.3	43	0
pJS54	*ADE3*(1-666)–*SER2*	5.1	26	0

[a] See the footnote of Table 1.

Table 3
Stability of enzyme activities in *ade3 mis1* strain S40 carrying different plasmids

Plasmid	Carried *ADE3* allele	Half life in days of enzymatic ctivity[a] (days)			
		D	C	S	β-Gal
pJS8A	*ADE3*	42	22	>>90	–
pJS8A-A	*ADE3*(1-333)	–	21	–	–
pBJL1	*ADE3*(1-333)–*lacZ*	6	6	–	>>90
pJS54	*ADE3*(1-666)–*SER2*	2	2	–	–

[a] The S and β-Gal activities were stable in the period of experiment (3 months).'–' indicates no test because of no detectable enzyme activity.

tained almost half level of the D activity and one-third level of the C activity when compared to that with the plasmid pJS8A carrying the *ADE3* gene.

Another fusion gene *ADE3*(1-666)–*SER2* has been constructed by fusing the *ADE3*(1-666) encoding a truncated protein to the *SER2* gene encoding phosphoserine phosphatase. The S40 strain transformed with the multicopy plasmid pJS54 carrying the constructed *ADE3*(1-666)–*SER2* does not require adenine for growth. Enzyme assays show that it retained a low level of the D/C activities (Table 2).

One possible explanation for the recovery of the D/C activities in the S40 strain transformed with the plasmid pBJL1 and the D activity in the strain transformed with the plasmid pJS54 is that the C-terminal portions, β-galactosidase and phosphoserine phosphatase of the fusion protein protects the N-terminal D/C domain from the proteolysis at the C-terminus by extending the length of polypeptide. To investigate this possibility, we have compared the stability in vitro among the fusion proteins encoded by the *ADE3*(1-333)–*lacZ* gene and the *ADE3*(1-666)–*SER2* gene, the truncated protein encoded by the *ADE3*(1-333) gene, and the Ade3 protein. The enzyme stability was determined by examining the half life of the enzymatic activities in vitro (Table 3). The D/C activities of the fusion protein is less stable than those of the Ade3 protein. The C activity of the fusion protein is also less stable than that of the truncated protein, while the C activity of the truncated protein is as stable as that of the Ade3 protein. However, the β-galactosidase activity of the fusion protein and the S activity of the Ade3 protein were stable for 3 months. These results indicate that the retained D/C activities of the fusion protein are not due to the protection from proteolysis at the C-terminus by the extended length of the polypeptide added. Rather, they suggest that the decay of N-terminal D/C domain occurs from the N-terminus or middle portion of the domain, and the D/C activities of the fusion protein are retained by the contribution of the C-terminal end on the enzyme stability in vitro. This explanation is supported by the fact that the D/C activities of the Ade3 protein is higher and more stable than those of the *ADE3*(1-333)–*lacZ* fusion protein, although the C-terminal portion of the Ade3 protein is 403-amino acids shorter than that of the fusion protein. However, at this point, we cannot distinguish between the possibilities that: (1) the C-terminal domain of the Ade3 protein protects the activity of the N-terminal domain (D) from the presence of an endogenous proteolytic enzyme, or (2) it is required for stabilizing the active conformation of the N-terminal domain.

These results demonstrate that the N-terminal D/C domain of the yeast cytoplasmic C1-THF synthase requires the C-terminal S domain for the catalytic activity in vitro, in contrast to the fact that both domains of the human C1-THF synthase form active enzymes and fold independently in *E. coli* [14]. The *ADE3*(1-333)–*lacZ* gene used in this study would be used to identify missense mutations on the D/C domain of *ADE3* gene, which cause the defectived catalytic activity and require adenine for growth. Such mutations could be contributed to clarify the relationship between the catalytic activity of C1-THF synthase and the purine biosynthesis.

Acknowledgements: We thank B.J. Lee for the construction of plasmid pBJL1. We thank D.A. Appling for the yeast strain CBY4. This work was supported by United States Public Health Services Grant 5 RO1DK02109-37.

References

[1] MacKenzie, R.E. (1984) in: Folates and Pterines, Vol. 1, pp. 256–306 (Blakley, R.L. and Benkovic, S.J., Eds.) John Wiley and Sons, New York.
[2] Dev, I.K. and Harvey, R.J. (1982) J. Biol. Chem. 257, 1980–1986.
[3] Whitehead, T.R., Park, M. and Rabinowitz, J.C. (1988) J. Bacteriol. 170, 995–997.
[4] Paukert, J.L., Williams, G.R. and Rabinowitz, J.C. (1977) Biochem. Biophys. Res. Commun. 77, 147–154.
[5] Uyeda, K. and Rabinowitz, J.C. (1967) J. Biol. Chem. 242, 4378–4385.
[6] O'Brien, W.E., Brewer, J.M. and Ljungdahl, L.G. (1973) J. Biol. Chem. 248, 403–408.
[7] Dev, I.K. and Harvey, R.J. (1978) J. Biol. Chem. 253, 4245–4253.
[8] Staben, C. and Rabinowitz, J.C. (1986) J. Biol. Chem. 261, 4629–4637.
[9] Shannon, K.W. and Rabinowitz, J.C. (1988) J. Biol. Chem. 263, 7717–7725.
[10] Hum, D.W., Bell, A.W., Rozen, R. and MacKenzie, R.E. (1988) J. Biol. Chem. 263, 15946–15950.
[11] Thigpen, A.E., West, M.G. and Appling, D.R. (1990) J. Biol. Chem. 265, 7907–7913.
[12] Tan, L.U.L. and MacKenzie, R.E. (1977) Biochim. Biophys. Acta 485, 52–59.
[13] Villar, E., Schuster, B., Peterson, B. and Schirch, V. (1985) J. Biol. Chem. 260, 2245–2252.
[14] Hum., D.W. and MacKenzie, R.E. (1991) Protein Eng. 4, 493–500.
[15] Song, J.M. and Liebman, S.W. (1989) Curr. Genet. 16, 315–321.
[16] Song, J.M. and Rabinowitz, J.C. (1993) Proc. Natl. Acad. Sci. USA 90, 2636–2640.
[17] Barlowe, C.K. and Appling, D.R. (1990) Mol. Cell. Biol. 10, 5679–5687.
[18] Rothstein, R.J. (1983) Methods Enzymol. 101, 202–211.
[19] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Casadaban, M.J., Chiou, J. and Cohen, S.N. (1980) J. Bacteriol. 143, 960–971.
[21] Suarez de Mata, Z. and Rabinowitz, J.C. (1980) J. Biol. Chem. 255, 2569–2577.
[22] Miller, J.H. (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] McKenzie, K.Q. and Jones, E.W. (1977) Genetics 86, 85–102.