ROLE OF THE 5'-TERMINAL SEQUENCE IN THE RNA BINDING SITE OF YEAST 5.8 S rRNA

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1. Introduction

The larger subunits of cytoplasmic ribosomes from eukayotic cells contain 5 S and 5.8 S rRNA; the 5.8 S rRNA molecule is hydrogen bonded to 26 S rRNA, in yeast [1–3]. The 5.8 S–26 S rRNA complex may be dissociated by heat, urea or aqueous denaturation [1–4] and easily reformed under appropriate salt conditions [4]. The 5.8 S rRNA may be specifically released from whole 60 S subunits with heat or formamide [5,6] and, at least in yeast, the molecule will reassociate with the subunit if the ionic strength is elevated [6]. Although the function of 5.8 S rRNA is unknown, the 5.8 S–26 S rRNA interaction is an integral part of the ribosome and is, therefore, likely to be important in the molecule's role in ribosome structure or function. Furthermore, because the complex can be isolated easily and well defined it is a good model for the RNA–RNA interactions which occur in the ribosome and which are likely to be basic to its function.

Although the sequence of a number of 5.8 S RNA species is now known [7] and a generalized model for their secondary structure in solution ('burp gun' model) has been proposed [8], the interaction between the 5.8 S RNA molecule and its cognate high molecular component is not fully understood. In mouse L-cells, the 3'-terminal 20–21 nucleotides of the 5.8 S RNA are associated with the 28 S rRNA [9] but thermal denaturation studies indicated that other nucleotides may be involved. Here, evidence is presented which indicates that the 5'-terminal sequence of the 5.8 S RNA molecule plays an important role in the 5.8 S–26 S RNA interaction and suggests that the junction may involve both ends of the 5.8 S RNA molecule.

2. Experimental

Saccharomyces cerevisiae, strain S288C, grown aerobically in 1% bacto-yeast extract, 2% bacto-peptone, and 2% glucose at 28°C were harvested in late log phase. Ribosomes and 60 S subunits were prepared essentially as in [10] and non-radioactive RNA was prepared by suspending the pellets in buffer (pH 5.1) containing 0.3% (w/v) sodium dodecyl sulfate (SDS) and extracting with an equal volume of phenol solution [11] at room temperature. The extracted RNA was dissolved in water and the 26 S rRNA component was purified by centrifugation at 200 000 × *g* for 12 h.

For ^{32}P-labeled RNA, cells were labeled in low phosphate medium [12] with 2 mCi carrier-free [^{32}P]-orthophosphate and whole cell RNA was extracted with the SDS–phenol method at 65°C [11]. The RNA was fractionated by polyacrylamide gel electrophoresis on an 8% slab at pH 8.3 and the low molecular weight components were recovered by homogenizing the gel [6,8].

Large fragments of 5.8 S rRNA were prepared by limited pancreatic ribonuclease digestion. [^{32}P]RNA (20–50 μg) was incubated on ice for 10–20 min in 10 μl buffer (1 mM EDTA, 10 mM Tris–HCl (pH 7.5)) containing 1 ng enzyme; the digest was mixed with an equal volume of formamide, loaded directly on a precooled 8% polyacrylamide slab gel and fractionated by electrophoresis at 4°C [8]. The fragments were detected by autoradiography and recovered by homogenizing the gel in water. For nucleotide sequence analyses, the fragments were further completely digested with pancreatic or T_1 ribonuclease; the digests were separated by one dimensional electrophoresis on DEAE-paper in 7% formic acid or two-dimensional electrophoresis on cellulose acetate at

pH 3.5 and DEAE-paper in 7% formic acid [13]. The nucleotide sequence was determined by comparing the oligonucleotides which were obtained with the known nucleotide sequence of yeast 5.8 S RNA [12]. The 3′-terminal sequence U–C–A–U–U–U was confirmed by the 'diagonal' method of Dahlberg [14].

The 5′-terminal sequence and other T_1 ribonuclease digestion fragments of yeast 5.8 S or 5 S RNA were prepared by incubating [^{32}P]RNA (10–20 μg) for 60 min in 20 μl buffer (1 mM EDTA, 10 mM Tris–HCl (pH 7.5)) containing 2 μg enzyme. The digest was loaded on an 20% polyacrylamide slab gel (10 × 40 cm) and fractionated by electrophoresis for 5 h at 1000 V [15]. The oligonucleotides were detected by autoradiography and recovered by homogenization and ethanol precipitation [6,8].

5.8 S rRNA or fragments were reconstituted into complexes with 26 S RNA by dissolving the RNA components in 20 μl water (0.5–1 A_{260} units of 26 S rRNA), adding an equal volume of 0.8 M KCl, 12 mM magnesium acetate, 10 mM β-mercaptoethanol, and 50 mM Tris–HCl (pH 7.5) and incubating the mixture for 30–60 min* at 0°C. Complex formation was usually evaluated by electrophoresis and 8% gel slabs [6]. Gels for separating complexes with T_1 ribonuclease digestion fragments were prepared by adding 1 vol. high salt buffer (0.8 M KCl, 12 mM magnesium acetate, 10 mM β-mercaptoethanol and 50 mM Tris–HCl (pH 7.5)) to 4 vol. gel mixture [8]; electrophoresis was carried out in buffer containing 1 vol. high salt buffer and 4 vol. standard electrophoresis buffer [8]. The thermal denaturation profiles of native or reconstituted complexes were examined by heating samples dissolved in the reconstitution buffer (0.4 M KCl, 6 mM magnesium acetate, 5 mM β-mercaptoethanol, and 25 mM Tris–HCl (pH 7.5)) at appropriate temperatures for 3 min and cooling them rapidly. The release of 5.8 S rRNA was determined by gel electrophoresis [6]; the RNA fractions were quantitated by staining the gels with methylene blue [6] and scanning them in a Gilford 240 spectrophotometer at 570 nm.

* The 5.8 S–26 S complex appeared to form within seconds of incubation; however, the incubation time was extended to ensure that there was a maximum opportunity for complexes to form with each of RNA species that were examined

3. Results and discussion

The generalized (burp gun) model for free 5.8 S rRNA [8] suggests that with its 3′-end engaged in the 5.8 S–28 S RNA interaction [9], the 5′-terminal sequence is the only sequence of significant length for a second RNA–RNA interaction. To explore this possibility, we investigated the effect of removing the 5′-end of yeast 5.8 S rRNA on the molecule's ability

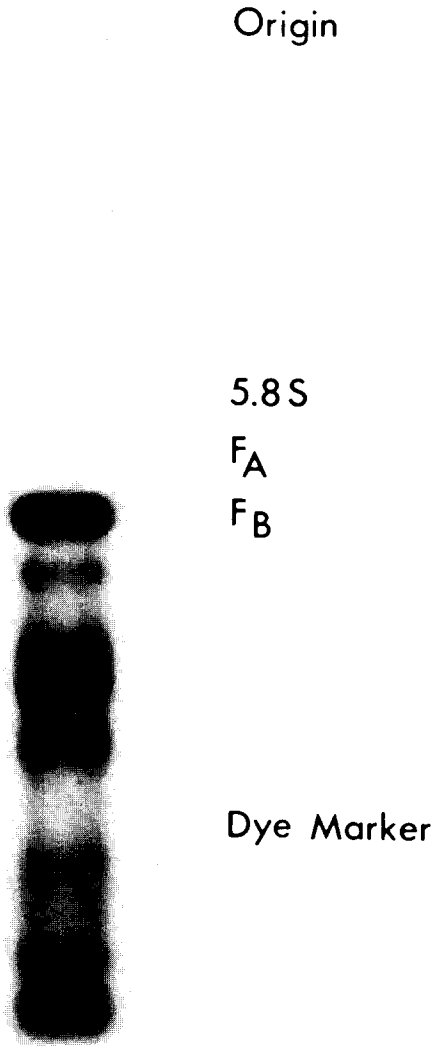

Fig.1. Fractionation of a limited pancreatic ribonuclease digest of ^{32}P-labeled 5.8 S rRNA from *Saccharomyces cerevisiae*. A 20 μg sample of RNA was digested in 10 μl buffer (1 mM EDTA, 10 mM Tris–HCl (pH 7.5)) with 1 ng enzyme for 15 min at 0°C, and the fragments were fractionated directly on an 8% polyacrylamide slab gel at pH 8.3 with 35 mA for 4 h. The positions of the origin and the bromophenol blue marker dye are indicated beside the autoradiograph.

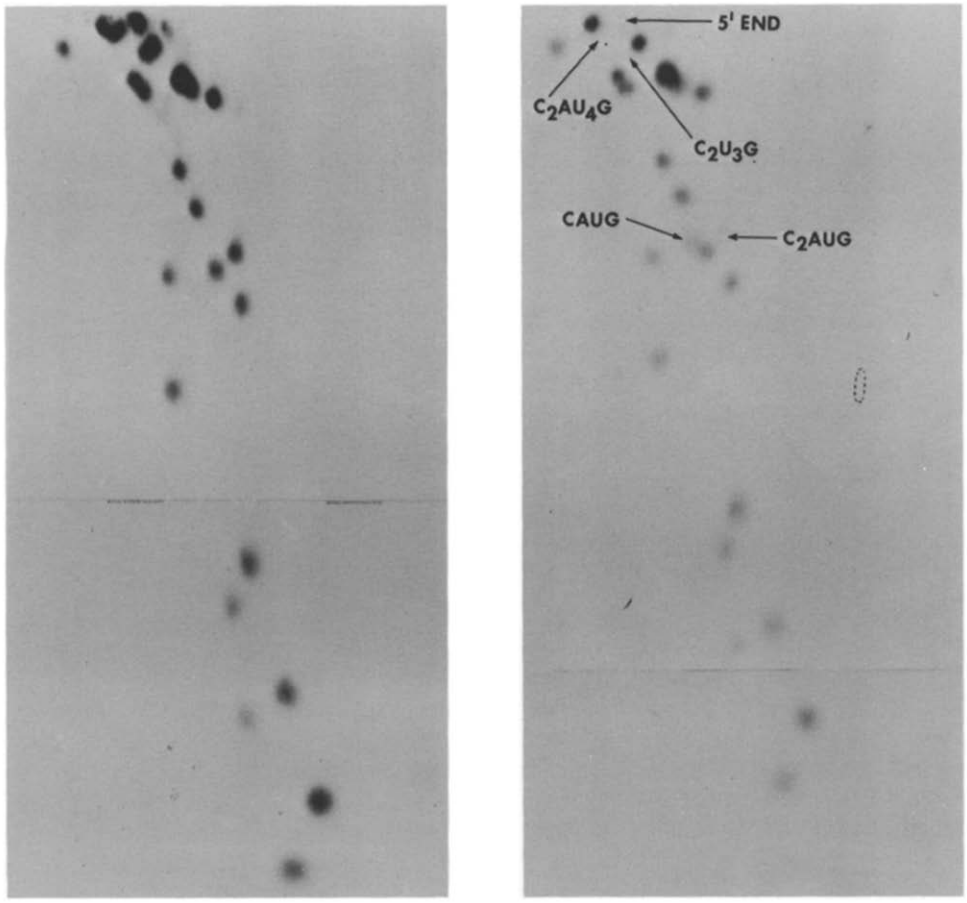

Fig.2. Fractionation of complete T_1 ribonuclease digests of ^{32}P-labeled 5.8 S rRNA (left) and fragment F_B (right) from *Saccharomyces cerevisiae*. Electrophoresis was, from left to right on cellulose acetate at pH 3.5, and from top to bottom on DEAE-paper in 7% formic acid. New or missing oligonucleotides are identified for the F_B fragment.

Table 1
Oligonucleotide sequences removed by partial digestion with pancreatic RNase

Band	Pancreatic RNase digestion products	T_1 RNase digestion products	Sequence
F_A	pA_3Cp, A_2Cp, Cp^a, $Up^a_{(3)}$	$pA_7C_4U_3Gp$	pA–A–A–C–U–U–U–C–A–A–Cp
F_B	AG_2Up, G_2Up, pA_3Cp, GCp, AUp^b, $A_2Cp_{(2)}Cp^a_{(5)}Up^a_{(8)}$	$pA_7C_7U_3Gp$, C_2AU_4Gp, C_2U_3Gp, C_2AUGp^b	pA–A–A–C–U–U–U–C–A–A–C–A–A–C–G–C–A–U–C–U–C–U–U–G–G–U–U–C–U–C–G–C(–A–Up)

[a] The molar yield of this product was based on the T_1 RNase digestion products

[b] Although the C–A–U–C–Gp product was essentially absent, significant amounts of a new oligonucleotide product, A–U–C–Gp, were observed indicating that fragment F_B was heterogeneous containing an extra A–Up sequence in about half the molecules

After the partial digests were fractionated by gel electrophoresis (e.g., fig.1) bands F_A and F_B were further analyzed by complete digestion with pancreatic or T_1 ribonuclease; the products were subsequently separated by one- or two-dimensional electrophoresis (e.g., fig.2). The overall sequences were deduced by arranging the end products of complete digestion which were absent along the known nucleotide sequence of yeast 5.8 S RNA [12]

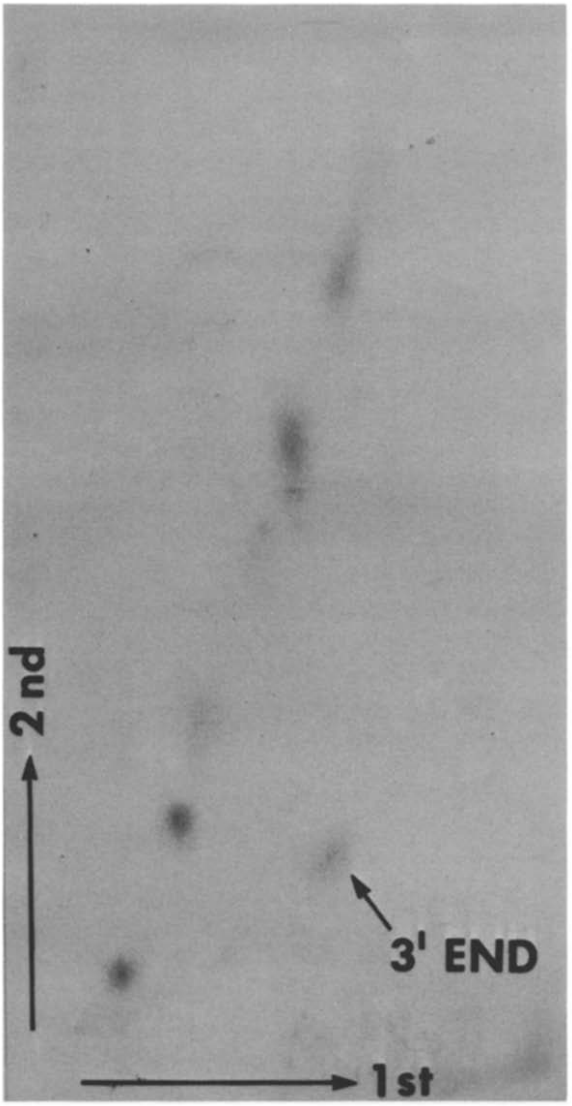

Fig.3. A diagonal fractionation of a T_1 ribonuclease digest of ^{32}P-labeled fragment F_B, treated with alkaline phosphatase between the two dimensions. Electrophoresis was on DEAE-paper in 7% formic acid; the 3'-terminal fragment was identified as the only spot migrating on the diagonal.

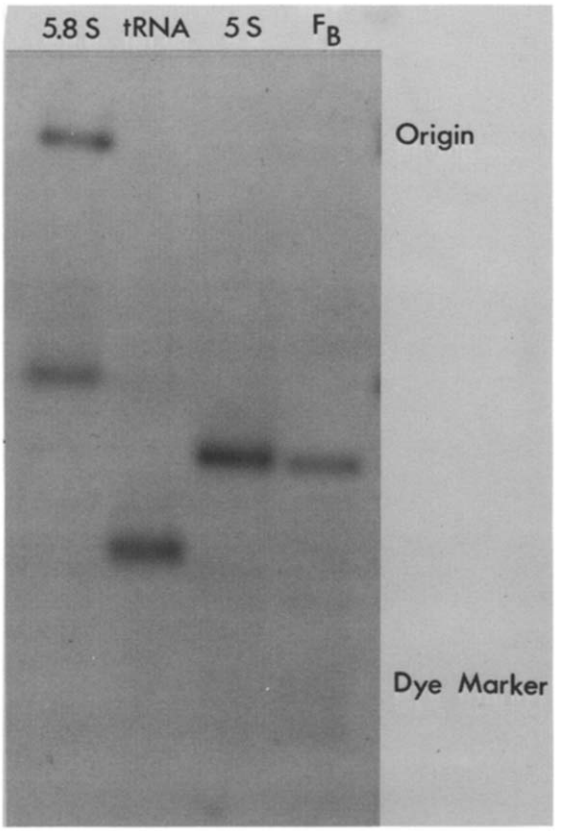

Fig.4. Reassociation of ^{32}P-labeled 5.8 S rRNA or pancreatic ribonuclease digestion fragment F_B into complexes with 26 S rRNA from *Saccharomyces cerevisiae*. ^{32}P-labeled yeast 5.8 S, 5 S, or tRNA or the F_B fragment (0.1–0.2 $A_{260\,nm}$ unit) were incubated with 1.0 $A_{260\,nm}$ unit of unlabeled 26 S rRNA in 40 liters 0.4 M KCl, 6 mM magnesium acetate, 5 mM β-mercaptoethanol, and 25 mM Tris–HCl (pH 7.5) for 30 min at 0°C and fractionated directly on an 8% polyacrylamide slab gel at pH 8.3. The positions of the origin and the bromophenol blue marker dye are indicated beside the autoradiograph.

to interact with its cognate high molecular weight RNA component. When yeast 5.8 S rRNA was digested with pancreatic ribonuclease under very mild conditions, two long fragments (F_A, F_B) were consistently observed (fig.1) when the products were analyzed by gel electrophoresis. Further analyses after complete pancreatic or T_1 ribonuclease digestion (fig.2, table 1) indicated that these were fragments from which portions of the 5'-end had been removed. To ensure that the 3'-end was present, the U–C–A–U–U–U terminal sequence was confirmed (fig.3) by a 'diagonal' analysis method [14] after treatment with alkaline phosphatase.

A number of methods have been reported by which 5.8 S RNA can be reconstituted into a complex with its cognate high molecular weight component [4]. In yeast, this may be accomplished by simply increasing the salt concentration, a method used to reconstitute yeast 5.8 S into 60 S ribosomal subunits [6]. As shown in fig.4, >60% of the RNA reassociated with the 26 S rRNA component when the RNA were incubated in 0.4 M KCl, 6 mM mag-

nesium acetate, 25 mM Tris–HCl (pH 7.5) and 5 mM β-mercaptoethanol at 4°C. In some laboratories higher temperatures have been used to reassociate 5.8 S RNA with their cognate high molecular weight components; in this case a brief incubation at 65°C actually significantly reduced the level of association. The association was very specific; other low molecular weight RNA such as yeast tRNA or 5 S RNA did not associate with the 26 S RNA molecule under these salt conditions (fig.4). Furthermore, thermal denaturation studies confirmed that this reassociation was native. As shown in fig.5, the denaturation profile of the reconstituted complex was essentially identical with that of the native complex. Although only ~60% of the 5.8 S RNA was reconstituted and present in complex form initially (e.g., 35°C), the T_m (47°C) for the associated fraction was equal to that of the native complex.

When fragments F_A and F_B were incubated with 26 S RNA, little or no complex was formed. As shown in fig.4, fragment F_B migrated as free RNA and no radioactivity was present at the origin where 26 S RNA or the 5.8 S–26 S RNA complex was retained. The results clearly show that the 5′-terminal sequence is essential for complex formation; either the sequence interacts directly with 26 S RNA or it is required for secondary structure which is itself essential for the 5.8 S–26 S RNA junction.

Much of the 5′-terminal sequence is represented by a single long T_1 ribonuclease digestion product,

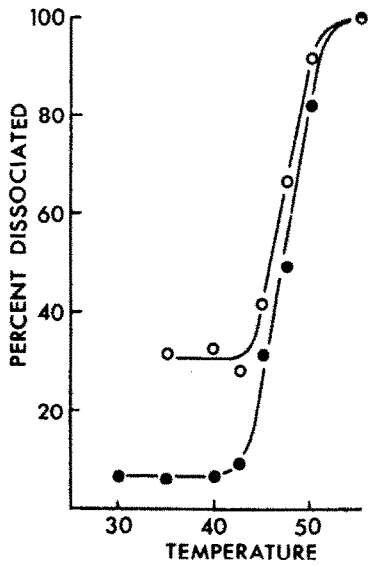

Fig.5. Dissociation of native and reconstituted 5.8 S–26 S rRNA complexes by thermal denaturation. Samples were prepared and incubated at various temperatures as in section 2. Free 5.8 S rRNA was fractionated by electrophoresis on an 8% polyacrylamide gel slab and quantitated by scanning the gels at 570 nm.

pAAACUUUCAACAACGp. Since this could represent an essential binding sequence, an attempt was made to reassociate this oligonucleotide with 26 S rRNA. When complexes were assessed by electrophoresis under the conditions in [6] little or no association

Table 2
Association of ^{32}P-labeled T_1 ribonuclease digestion products with 26 S rRNA

Fragment	Source	% Radioactivity associated
Undigested	5.8 S RNA	64.5%
pAAACUUUCAACAACGp	5.8 S RNA	13.8%
Octanucleotide fraction	5.8 S RNA	3.6%
Ethanol-insoluble fraction	5.8 S RNA	2.2%
Ethanol-insoluble fraction	5 S RNA	1.8%

T_1 ribonuclease digests of ^{32}P-labeled 5 S or 5.8 S RNA were extracted with SDS–phenol [11] and the larger fragments were ethanol precipitated. Aliquots of each digest were fractionated on a 20% polyacrylamide slab gel at pH 8.3 using 1000 V for 5 h [15]. Equivalent molar amounts of each fraction (as determined from the radioactivity) were dissolved in 20 μl water containing 25 μg yeast 26 S RNA, mixed with an equal volume of high salt buffer, and incubated on ice for 60 min. Percentage of radioactivity associated with the 26 S RNA molecule was determined after further fractionation on a 6.4% polyacrylamide gel using 30 V for 6 h

was observed. However, as shown in table 2, when the experiments were repeated using a higher ionic strength during electrophoresis, a limited association was observed between the 5′-terminal sequence of 5.8 S RNA and the 26 S RNA molecule. Although less efficient and less stable than observed with whole 5.8 S RNA, the complex between the 5′-terminal fragment and 26 S RNA still appeared relatively specific. Other 5.8 S RNA fragments did not associate to a comparable degree and no significant association was observed with fragments of 5 S RNA (table 2). Further studies such as crosslinking experiments will be required to confirm this direct interaction. Nevertheless, although the exact binding sites are not fully defined, based on [9] and this study, it is likely that 5.8 S rRNA interacts with its cognate high molecular weight RNA component through at least 2 sites localized in its 5′- and 3′-terminals. As yet nothing is known of the site of interaction in the cognate high molecular weight RNA component. The ease with which 5.8 S RNA reassociates indicates that the site must be readily available and efforts are currently being made to identify the RNA sequences which are involved.

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References

[1] Forget, B. G. and Weissman, S. M. (1967) Nature 213, 878–882.
[2] Pene, J. J., Knight, J. E. and Darnell, J. (1968) J. Mol. Biol. 33, 609–623.
[3] Weinberg, R. A. and Penman, S. (1968) J. Mol Biol. 38, 289–304.
[4] Oakden, K. M. and Lane, B. G. (1973) Can. J. Biochem. 51, 520–528.
[5] Giorgini, J. F. and De Lucca, F. L. (1976) Nucleic Acid Res. 3, 165–175.
[6] Nazar, R. N. (1978) J. Biol. Chem. 253, 4505–4507.
[7] Nazar, R. N. and Roy, K. L. (1978) J. Biol. Chem. 253, 395–399.
[8] Nazar, R. N., Sitz, T. O. and Busch, H. (1975) J. Biol. Chem. 250, 8591–8597.
[9] Pace, N. R., Walker, T. A. and Schroeder, E. (1977) Biochemistry 16, 5321.
[10] Torano, A., Sandoval, A. and Heredia, C. F. (1974) Methods Enzymol. 30, 254–261.
[11] Steel, W. J., Okamura, N. and Busch, H. (1965) J. Biol. Chem. 240, 1742–1749.
[12] Rubin, G. M. (1973) J. Biol. Chem. 248, 3860–3875.
[13] Sanger, F. and Brownlee, G. G. (1967) Methods Enzymol. 12, 368–381.
[14] Dahlberg, J. E. (1968) Nature 220, 548–549.
[15] Sanger, F. and Coulson, A. R. (1978) FEBS Lett. 87, 107–110.
[16] Valenzuela, P., Bell, G. I., Masiarz, F. R., DeGennaro, L. J. and Rutter, W. J. (1977) Nature 267, 641–643.