THE ROLE OF RIBOSOMAL RNA IN PROTEIN SYNTHESIS

Inhibition of translation by reticulocyte 5 S ribosomal RNA

D. H. WRESCHNER

Division of Clinical Investigation, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, England

Received 14 July 1978

1. Introduction

Understanding the functions of the integral parts that compose the ribosome is an essential prerequisite for elucidating the complex regulatory mechanisms that control protein synthesis. It is now obvious that the interplay between the ribosome and cytoplasmic as well as membrane components may significantly affect the protein synthetic capability of the ribosomal machinery. Factors required for the initiation, elongation and release of the peptide chain transiently oscillate between the ribosome and the soluble cytoplasm [1,2]. The presence or absence of these molecules in the immediate vicinity of the ribosome will profoundly affect ribosomal function.

Thus, although it is clear that changes in the environment directly adjacent to the ribosome may influence pleiotypic ribosomal responses, such as the extent and rate of protein synthesis as well as selection of specific mRNAs for translation [3], the role that the ribosome, as a functional integrity, plays in the regulation of protein synthesis is both neglected and poorly understood.

It is to be expected that the multitude of ribosomal proteins are not only structurally important but also perform functions that are essential for correct ribosomal activity. For example, ribosomes which are streptomycin resistant display a different ribosomal-protein composition to sensitive ribosomes [4]. This seems to indicate that a significant relationship exists between ribosomal structure and function.

Information on the significance of ribosomal RNA in determining ribosomal function is surprisingly scant. Indirect evidence has implicated 18 S rRNA in the recognition and/or initiation stages of protein synthesis [5,6]. Moreover, the structural integrity of ribosomal RNA has, in certain cases, been found to be critical for correct ribosomal functioning. Thus, colicin E3 treatment inactivates bacterial ribosomes by cleaving specifically a fragment 50 nucleotides in length in the 16 S rRNA of the 30 S subunit [7]. Similar observations as to the importance of structural integrity of rRNA have been made in eukaryotic cells. Alpha sarcin, a potent inhibitor of eukaryotic protein synthesis cleaves, specifically, rRNA on the 60 S subunit of the ribosome and in so doing inhibits elongation of the peptide chain [8,9]. In addition, it has been shown recently that the reticulocyte cell membrane harbours an endonucleolytic enzyme that strongly inhibits protein synthesis by cleaving specifically the 28 S rRNA of the 60 S subunit [10,11].

These findings demonstrate that changes both in the ribosomal protein make-up as well as in the structural integrity of the RNA can modify ribosomal behaviour. Controls on the regulation of translation are, therefore, not only restricted to cytoplasmic and/or membrane interactions with the ribosome but also by the ribosome itself.

The mammalian ribosome contains in addition to 28 S and 18 S RNA, 5 S, 5.8 S [12] and 7 S [13] RNA species. The functions of all these molecules are relatively unknown. This work uses a novel approach to gain insight into the possible functions of ribosomal RNA. It is found that whereas 28 S and 18 S RNA have little effect on protein synthesis, the addition of small amounts of 5 S RNA causes profound inhibition

of protein synthesis. It is also reported here that this potent inhibition may in some way be related to the double-stranded nature of the 5 S RNA molecule.

2. Materials and methods

Reticulocyte ribosomes were prepared from rabbit reticulocytes as in [10]. Ribosomal RNA was isolated from ribosomes either by phenol–chloroform extraction [10] or by the proteinase K method. When the latter method was used, ribosomes were suspended at 5 mg/ml in a buffer containing 15 mM KCl, 10 mM Tris–HCl (pH 7.4) and 1.5% SDS. Proteinase K (British Drug Houses) was added to a final concentration of 0.2 mg/ml and the suspension incubated at 25°C for 30 min. Two volumes of cold ethanol were added and the RNA was allowed to precipitate overnight at −20°C. The precipitated RNA was spun down by a 10 min centrifugation at 10 000 rev./min and the pellet resuspended in ethanol–0.2 M NaCl (2:1), then recentrifuged. This process was repeated 3 times. The RNA pellet was dried, resuspended in distilled water and kept at −70°C.

The individual ribosomal RNA species were isolated from total ribosomal RNA by preparative sucrose gradient. Ribosomal RNA, 2 mg, was loaded onto 60 ml of a 8–20% isokinetic exponential sucrose gradient made up in 100 mM KCl, 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA. The tubes were spun in an MSE Superspeed 65 centrifuge with the MSE 3 × 65 ml rotor for 22 h, 22 500 rev./min at 4°C. Fractions were collected, their absorbance determined and 2 vol. absolute ethanol was added to the peak fractions corresponding to 28 S, 18 S and 5 S RNA. The RNA precipitated overnight and was collected by a 10 min centrifugation at 10 000 rev./min. The RNA pellet was dried, resuspended in distilled water and kept at −70°C.

Globin 9 S messenger RNA was prepared by passing 10 mg of total polysomal RNA (prepared as above) through a short column (2 × 0.5 cm) of poly(U)–Sepharose (Pharmacia Products) equilibrated in 300 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% SDS and continuing elution at 25°C with this buffer until the absorbancy of eluting fractions reached background levels. The buffer was then changed to 10 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% SDS and elution was continued at 25°C. Fractions were collected and the ultraviolet absorbancy checked. When the absorbancy reached background levels the column itself and the eluting buffer were heated to 50°C. Elution was continued at 50°C. The RNA that eluted with the low ionic strength buffer both at room temperature and at 50°C was precipitated by adding 2 vol. cold ethanol at −20°C. The RNA was collected as described above. Analytical gradient centrifugation showed that RNA eluting at 25°C was 20% 9 S globin mRNA, whereas that eluting at 50°C consisted of 40–50% 9 S mRNA (data not shown).

The wheat germ embryo lysate system was as in [14]. Incorporation of [^{35}S]methionine into protein was performed as in [10] using cold 10% trichloroacetic acid (TCA) precipitation onto glass filters (GF/C, Whatman) followed by 90°C, 5% TCA with subsequent washes in 5% cold TCA, ethanol–ether (1:1), ether and drying.

Analysis of ribosomal RNA species was performed on 8–20% isokinetic exponential sucrose gradients made up in 100 mM KCl, 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA. Centrifugation was for 150 min, 48 000 rev./min at 4°C using an MSE 6 × 5 ml rotor in the MSE Superspeed 65 centrifuge.

Ribosomal populations were analysed using 15–40% isokinetic exponential sucrose gradients made up in 80 mM KCl, 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and 0.1 mM EDTA. The 5 ml tubes (MSE 6 × 5 ml rotor) were spun for 60 min, 48 000 rev./min at 4°C in an MSE Superspeed 65 centrifuge.

3. Results

Ribosomal RNA was extracted from isolated reticulocyte ribosomes using the proteinase K–SDS method as outlined above. The individual RNA species were obtained by separating total ribosomal RNA on preparative sucrose gradients and the isolated RNA species were analysed for their purity by analytical sucrose gradient centrifugation. Figure 1 shows that whereas the 28 S RNA component was homogeneous (fig.1A), the isolated 18 S RNA species was slightly contaminated in the lower molecular weight region with minute amounts of 5 S RNA (fig.1B). The isolated 5 S rRNA component was ~95% pure (fig.1C).

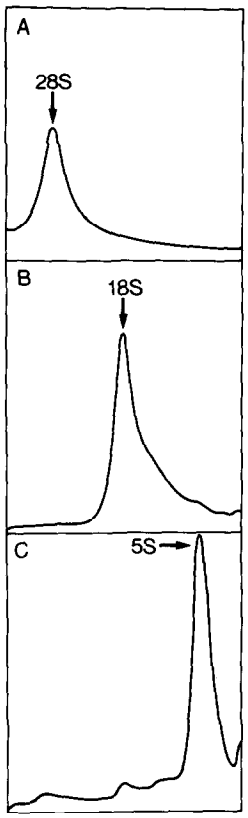

Fig.1. The individual ribosomal RNA species were isolated from rabbit reticulocyte ribosomal RNA as described in section 2. Of each species (28 S, 18 S or 5 S) ~4 μg was placed on an isokinetic exponential 8–20% sucrose gradient and centrifuged for 150 min at 48 000 rev./min. The gradients were pumped through a Unicam SP500 spectrophotometer using a flow cell with a 0.1 cm light path. The A_{260} profile was simultaneously recorded on an external recorder. (A) Isolated 28 S rRNA; (B) isolated 18 S rRNA; (C) isolated 5 S rRNA. Sedimentation is from right to left.

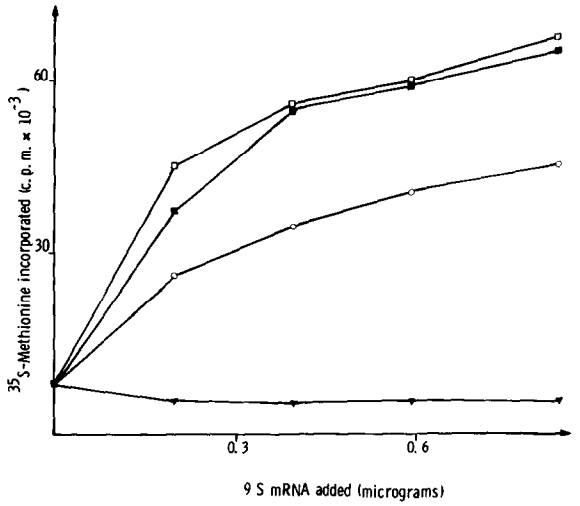

Fig.2. The wheat germ lysate system was set up as described in section 2 and the incorporation of [^{35}S]methionine into hot TCA-precipitable radioactivity was assayed after the tubes had been incubated for 90 min at 25°C. Protein synthesis was determined with increasing amounts of 9 S mRNA alone (■———■), in the presence of 4 μg of isolated 28 S rRNA with increasing amounts of 9 S mRNA (□———□), in the presence of 2 μg of isolated 18 S rRNA with increasing amounts of 9 S mRNA (○———○), or finally (▼———▼) with 0.25 μg of 5 S rRNA and increasing amounts of 9 S mRNA. The final volume of the protein synthesizing system was 50 μl/tube.

To investigate the possible effects of these isolated ribosomal RNA species on protein synthesis, a simple direct approach was adopted. The different individual RNA types were added to a protein synthesizing system composed of a wheat germ embryo lysate. The effect of adding these RNA molecules on the wheat germ lysate translation of exogenously supplied 9 S globin mRNA was investigated. In the control system (fig.2) the translation by the wheat germ lysate responded linearly to increasing amounts of 9 S globin mRNA up to 0.4 μg, after which the linear response gradually curved off. At the maximum quantity of mRNA added the wheat germ showed an 800% stimulation of translation over background levels. RNA, 28 S, 18 S or 5 S, was added individually to the wheat germ system at final concentrations equimolar with 0.4 μg of reticulocyte globin 9 S mRNA. The addition of 28 S RNA did not cause any appreciable change in the extent of protein synthesis (fig.2) and at all concentrations of 9 S mRNA tested the addition of 28 S rRNA did not cause any significant deviation from the control response. The addition of 18 S RNA (2 μg) caused only slight inhibition (max. 30%) in the translational capacity of the wheat germ embryo system. This inhibition could, however, be due to minor contamination of the 18 S RNA with 5 S RNA molecules. In sharp contrast to the results obtained both with 28 S and 18 S RNA, the addition of as little as 0.25 μg isolated 5 S rRNA, completely inhibited all translation. This 5 S RNA inhibitory

effect on translation was seen at all tested concentrations of 9 S mRNA.

Results similar to the above were also obtained when a phenol–chloroform procedure was used to isolate the ribosomal RNA species. In this case, only the 5 S RNA molecules caused considerable inhibition in the translation of exogenous 9 S mRNA by the wheat germ system (data not shown). Thus, the possibility that proteinase K contaminants of the 5 S RNA preparation were responsible for the inhibitory effect was excluded.

The effect of adding increasing concentrations of isolated 5 S RNA to the wheat germ embryo system is shown in fig.3. The translation of the exogenously added 9 S mRNA was 54% inhibited at 1.2 μg/ml 5 S RNA. This represents a molar ratio of ~1.7 molecules

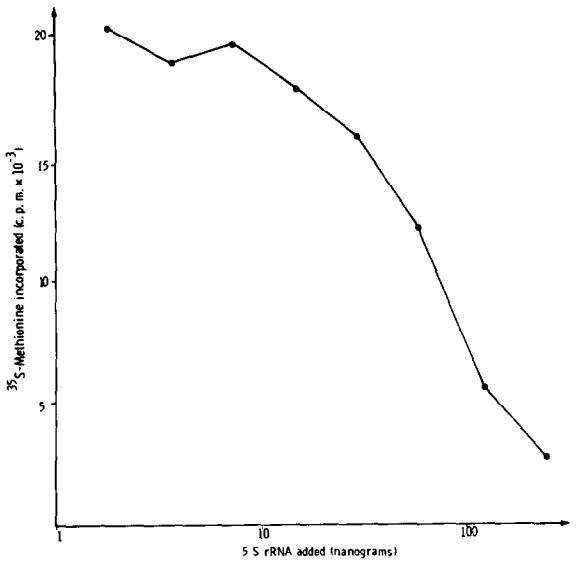

Fig.3. The wheat germ lysate system was set up as described in section 2. The tubes had a final volume of 50 μl and were supplemented with 9 S mRNA at a final concentration of 4 μg/ml. Increasing concentrations of isolated 5 S rRNA were added to the system in order to assay the effect on the incorporation of [^{35}S]methionine into protein. Incorporation in the presence of 9 S mRNA (and absence of 5 S rRNA) was 20 546 cpm and in the absence of 9 S mRNA (background level) 5 267 cpm. Inhibition of protein synthesis is expressed by the formula:

$$100 - \left(\frac{\text{test}-\text{background}}{\text{control}-\text{background}} \times 100\right)$$

of 9 S mRNA for every one 5 S RNA molecule. When the proportion of 9 S mRNA:5 S RNA molecules was decreased to 0.8 (2.4 μg/ml 5 S RNA) the translation of exogenously added messenger RNA was inhibited by 97%. Higher concentrations of 5 S RNA totally eliminated the translation of exogenously added 9 S mRNA and even inhibited the low endogenous level of wheat germ protein synthesis.

The 5 S RNA extracted and subsequently isolated from reticulocyte ribosomes may have contained (although in small amounts) transfer RNA, aminoacylated with cold methionine. In such a way, the potent inhibition of protein synthesis exhibited by the 5 S RNA species, could conceivably have been due to simple dilution of the marker radioactive methionine. This possibility was investigated by observing the effect of 5 S RNA on the mRNA-dependent formation of polysomes. If the 5 S RNA species was causing a reduction in the levels of hot TCA-precipitable radioactivity due to competition with radioactive amino acid, then the observed 9 S mRNA-promoted formation of polysomes would not be affected. Figure 4 shows the results of such an experiment. The ribosomal population of the wheat germ lysate after a 20 min incubation at 25°C under optimum conditions for protein synthesis was composed only of 80 S monosomes, 60 S and 40 S subunits. Polysomes were completely absent (fig.4A). The addition of globin 9 S mRNA promoted the formation of polysomes – di-, tri-, tetra- and pentasomes were easily visible (fig.4B). The formation of polysomes was clearly due to the mobilization of both 80 S monosomes and 60 S and 40 S subunits – all these species were reduced in quantity as compared to the control system lacking 9 S mRNA (c.f. Fig.4A,4B). The effect of adding decreasing amounts of 5 S rRNA to the 9 S mRNA-supplemented wheat germ system is depicted in fig.4C–F. It is immediately apparent that at 9 S RNA : 5 S RNA molar ratios of 0.5 and 1 (fig.4C,D), the formation of polysomes is markedly inhibited. At the molar ratio of 0.5 (fig.4C) polysomal formation is reduced by almost 95% and is similar to the ribosomal profile obtained in the absence of 9 S mRNA (fig.4A). At higher 9 S : 5 S ratios (4:1 and 20:1), the inhibition of polysome formation is significantly reduced (fig.4E,F) although still slightly lower than the 9 S mRNA-supplemented control (fig.4B).

Thus, the possibility that 5 S rRNA inhibits protein

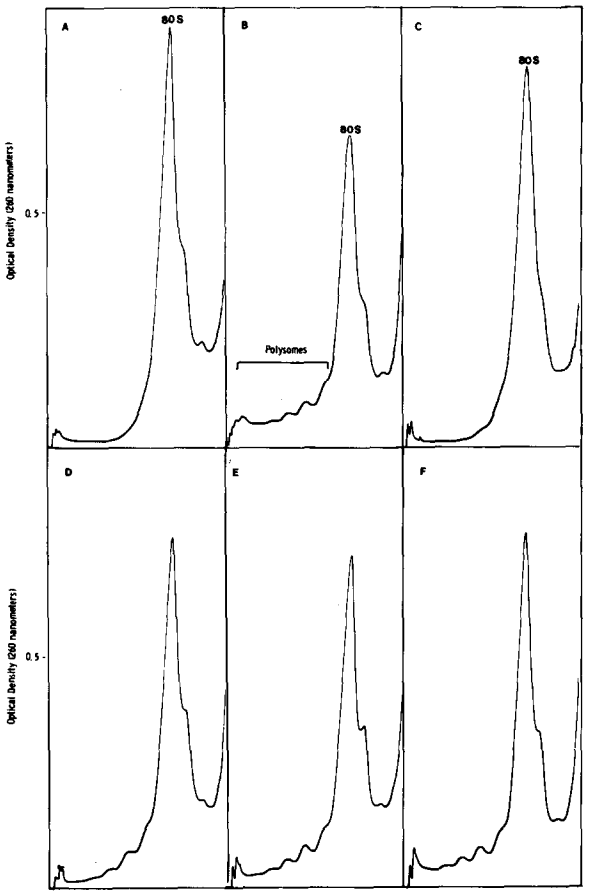

Fig.4. The wheat germ system was set up to give a final volume of 200 μl/incorporation test tube. The tubes were incubated for 20 min at 25°C, then chilled. The contents were placed on 15–40% isokinetic exponential sucrose gradients in 80 mM KCl, 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA and centrifuged 55 min at 48 000 rev./min and 4°C. The gradients were processed as described in fig.1 legend. (A) Wheat germ system without any additions; (B) wheat germ system with 4 μg of 9 S mRNA; (C) as B plus 4 μg of 5 S rRNA; (D) as B plus 2 μg of 5 S rRNA; (E) as B plus 0.5 μg of 5 S rRNA; (F) as B plus 0.1 μg of 5 S rRNA. Sedimentation is from right to left.

synthesis simply by diluting radioactive amino acid concentration was effectively excluded.

The wheat germ embryo system was supplemented with exogenous uncharged tRNA present at a final concentration of 75 μg/ml. This represents a concentration > 30-fold that required for total inhibition of protein synthesis by the 5 S rRNA. It is therefore not feasible that non-aminoacylated tRNA possibly present in the 5 S RNA preparation interfered with protein synthesis.

The effect of heating the 5 S rRNA on its inhibitory activity was next checked. Samples of the 5 S rRNA were kept for 4 min at varying temperatures, subsequently placed in ice and then added to a wheat germ embryo system supplemented with globin 9 S mRNA. Heating the 5 S rRNA at increasing temperatures caused a gradual decline in inhibitory activity (65%, 59% and 55% inhibition after heating 5 S RNA at 35°C, 45°C and 55°C, respectively, fig.5). Heating at 65°C caused the inhibition to fall further to 40% and treatment of the 5 S rRNA at 75°C almost completely abolished all inhibitory activity (fig.5).

These results demonstrate that heat denaturation destroys the inhibitory activity of the 5 S rRNA

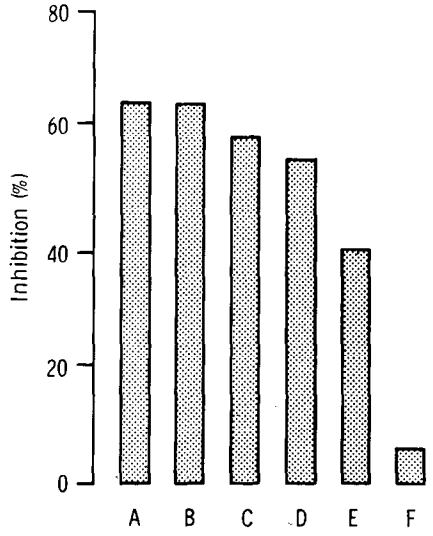

Fig.5. The wheat germ system was set up as described in section 2. Both 9 S mRNA and 5 S rRNA were added to the incorporation test tubes. The 9 S mRNA was added to a final concentration of 4 μg/ml. The 5 S rRNA used in this experiment was from a different batch to that used in fig.1,2 and was slightly less potent. The 5 S rRNA at a concentration of 28 μg/ml was heated for 4 min at the following temperatures: (A) kept in ice; (B) 35°C; (C) 45°C; (D) 55°C; (E) 65°C; (F) 75°C. The 5 S rRNA samples were added to the incorporation test tubes to give a final concentration of 5.5 μg/ml. Incorporation of [^{35}S]methionine into protein was tested as in section 2. The % inhibition was calculated as described in the fig.3 legend.

molecule. This indicates that the 5 S rRNA-induced inhibition on translation may be directly dependent on a certain molecular configuration involving hydrogen bonds.

4. Discussion

This work shows that 5 S rRNA, isolated and extracted from rabbit reticulocyte ribosomes, can profoundly inhibit protein synthesis. Inhibition of the translation of 9 S globin mRNA by the wheat germ lysate, was not found to be due to possible contaminants (such as tRNA, both aminoacylated and uncharged, or proteinase K) of the 5 S RNA preparation. The inhibitory activity must thus be attributed to the 5 S RNA molecule itself.

Besides 5 S ribosomal RNA, two other low molecular weight ribosomal RNA species have been reported [12,13]. A 7 S RNA molecule, about 130 nucleotides in length, was shown to be hydrogen bonded with the 28 S RNA molecule and to be derived from the same polynucleotide precursor as the 28 S ribosomal RNA [13]. Similar work showed [12] that a 5.8 S RNA species was also hydrogen bonded to the 28 S RNA molecule and could be released by heat treatment. These two reports in fact seem to be dealing with the same ribosomal RNA species. However, as the 5 S rRNA (described here) was extracted and isolated from ribosomes under conditions that do not break down hydrogen bonds, it would not be equivalent to either of these small rRNA species. Indeed, the 5.8 S rRNA species only appeared after isolated 28 S rRNA was heat treated for 4 min at 65°C (data not shown).

No reports have appeared on the possible functions of 5 S rRNA. The inhibitory activity of 5 S rRNA on protein synthesis, reported here, raises the possibility that the ribosome itself may contain self-regulatory mechanisms. Although the experiments described are distant to an actual in vivo situation, the results may shed light on the role that the 5 S rRNA plays in natural conditions within the cell.

It may be speculated that within the cell either aging of the ribosome or action of enzymes on the ribosome, may cause structural changes and thus free the 5 S rRNA from a previously constrained state, allowing it now to inhibit protein synthesis. Moreover, it has been shown (fig.5) that the inhibitory activity of 5 S rRNA may be critically dependent on a certain double-stranded RNA configuration. This raises the intriguing possibility that the 5 S rRNA may be a natural in vivo equivalent of artificial double-stranded RNA, such as poly (I)–poly(C), which has been shown to effect abrupt inhibition of protein synthesis both in reticulocyte lysates and in interferon-treated cells [15]. Further evidence supporting this proposition is in the accompanying paper [16].

Acknowledgements

D.H.W. is a recipient of an EMBO Long Term Fellowship. The author is grateful to Mrs Dorothy Seale and Mrs Patricia Davidson for their fine secretarial work.

References

[1] Lodish, H. F. (1976) Ann. Rev. Biochem. 45, 39–72.
[2] Nudel, U., Lebleu, B., Zehavi-Willner, T. and Revel, M. (1973) Eur. J. Biochem. 33, 314–322.
[3] Heywood, S. M. and Kennedy, D. S. (1976) Prog. Nucleic Acid Res. 19, 477–484.
[4] Gorini, L. (1969) Cold Spring Harb. Symp. Quant. Biol. 31, 101–111.
[5] Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. USA 71, 1342–1346.
[6] Steitz, J. A. and Jakes, K. (1975) Proc. Natl. Acad. Sci. USA 72, 4734–4738.
[7] Boon, T. (1971) Proc. Natl. Acad. Sci. USA 68, 2421–2425.
[8] Holden, A. N. and Cundliffe, E. (1978) Biochem. J. 170, 57–61.
[9] Schindler, D. G. and Davies, J. E. (1977) Nucleic Acid Res. 4, 1077–1110.
[10] Wreschner, D., Melloul, D. and Herzberg, M. (1978) Eur. J. Biochem. 85, 233–241.
[11] Wreschner, D., Melloul, D. and Herzberg, M. (1978) Eur. J. Biochem. in press.
[12] Pace, N. R., Walker, T. A. and Schroeder, E. (1977) Biochemistry 16, 5321–5328.
[13] Pene, J. J., Knight, E. and Darnell, J. E. (1968) J. Mol. Biol. 33, 609–623.
[14] Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330–2334.
[15] Hovanessian, A. G. and Kerr, I. M. (1978) Eur. J. Biochem. 84, 149–159.
[16] Wreschner, D. H. (1978) FEBS Lett. 94, 145–151.