

Inhibition of eukaryotic ribonuclease P activity by aminoglycosides: kinetic studies

Apostolos Tekos, Antigoni Tsagla, Constantinos Stathopoulos¹, Denis Drinas*

Department of Biochemistry, School of Medicine, University of Patras, 26500 Patras, Greece

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Abstract The effect of several aminoglycoside antibiotics on ribonuclease P (RNase P) was investigated using an in vitro experimental system from *Dictyostelium discoideum*. Detailed kinetic analysis showed that all aminoglycosides tested (tobramycin, gentamicin, kanamycin, paromomycin, neomycin) behave as classical non-competitive inhibitors, with neomycin being the strongest inhibitor. The inhibition effect is attributed to the electrostatic competition of the cationic aminoglycosides with magnesium ions required for catalysis. Increasing Mg^{2+} ion concentrations reduced the effect of aminoglycosides on RNase P activity. Detailed kinetic analysis showed that aminoglycosides compete with Mg^{2+} for common binding sites on RNase P holoenzyme. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: RNase P; Ribozyme; Antibiotic

1. Introduction

Antibiotics are produced by microorganisms as products of secondary metabolism, possessing the ability to kill or inhibit the growth of bacteria and other microorganisms. The discovery of the catalytic properties of RNA has brought RNA-binding antibiotics to proscenium. Most of them have ribosomal RNA as a target [1,2]. Also, many pathogen-specific RNAs, such as those of human hepatitis delta virus (HDV) and human immunodeficiency virus (HIV), are inhibited by certain antibiotics [3]. Inhibition of RNA biological activity can occur in various ways; by preventing binding of another macromolecule (protein or RNA), by distorting the RNA active conformation, by competitive binding for a cofactor binding site, or by displacing catalytically important ions, such as magnesium ions [4]. Aminoglycoside antibiotics are known to disturb the decoding process [1]. The decoding function of the ribosome is attributed to the small ribosomal subunit, and takes place at the ribosomal A-site, a region located near the 3' end of the 16S rRNA. The aminoglycoside antibiotics impair the decoding function by acting on this region [2]. These antibiotics, thought to act specifically on the ribosome, also act as inhibitors of catalytic RNAs, such as the self splic-

ing group I introns [5], the hammerhead ribozyme [6], the HDV ribozyme [7], the hairpin ribozyme [8], the HIV RNA [9] or *Escherichia coli* RNase P RNA [10].

The ribonucleoprotein RNase P is the endonuclease responsible for the maturation of 5' termini of tRNA molecules [11]. Divalent metal ions are essential for efficient chemical catalysis by RNase P. Among them, Mg^{2+} promotes cleavage most efficiently [12]. Recently, in a study on the metal ion requirements of RNase P from *Dictyostelium discoideum* we showed that there are two metal ion binding sites, one activating and another one inhibitory, and we proposed that one Mg^{2+} ion promotes the cleavage mechanism of *D. discoideum* RNase P [13]. In vitro, the RNA subunits of RNase P enzymes from bacteria [14] and from some archaea [15] are catalytically active in the absence of protein and are the only known RNA catalysts naturally devoted to act in *trans*. Similar behavior has not yet been demonstrated for eukaryotic RNase P enzymes. Although the latter, include an RNA subunit of similar size as found in their prokaryotic counterparts, they are composed of multiple protein components which contribute about 70% to the enzyme's molar mass [16]. This high protein/RNA ratio is a common feature of all eukaryotic nuclear RNase P enzymes characterized to date [11], including nuclear *D. discoideum* RNase P [17]. In addition, human orthologues of the yeast RNase P protein subunits have been found [18]. These findings indicate that the structures of RNase P enzymes from different eukaryotes are similar. Thus, *D. discoideum* RNase P is a good model system for other eukaryotic RNase P enzymes, such as the human nuclear RNase P, and could become a promising system to evaluate toxic side effects of antimicrobial inhibitors. Recently, we reported that natural and synthetic retinoids inhibit *D. discoideum* RNase P activity [19].

In the present study we examined the effect of several aminoglycosides on RNase P activity from *D. discoideum*. The influence of Mg^{2+} on aminoglycoside inhibition effect is also analyzed, and the molecular basis of this relationship is discussed.

2. Materials and methods

All chemicals were obtained from commercial sources. Aminoglycosides were obtained from Sigma.

2.1. Assay for RNase P activity

Enzyme assays were carried out at 37°C in 20 µl buffer D (50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 10 mM NH_4Cl) in the presence of 5 fmol labeled transcript of the *Schizosaccharomyces pombe* tRNA^{Ser} gene supS1 and 1.3 µg protein from the RNase P fraction, if not

*Corresponding author. Fax: (30)-61-997690.
E-mail: drinas@med.upatras.gr

¹ Present address: Department of Molecular Biophysics and Biochemistry, Yale University, 266 Whitney Av., P.O. Box 208114, New Haven, CT 06520-8114, USA.

stated otherwise. The reactions were stopped by addition of 5 μ l stop dye (80% formamide, 50 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Reaction products were resolved on 10% polyacrylamide/8 M urea gels and visualized by autoradiography without drying. Activity was quantified by Cerenkov counting of excised gel slices.

2.2. Enzyme purification

Growth of *D. discoideum* cells (strain AX2 wild type), cell breakage and the production of S-100 fraction were carried out as previously described [17]. The purification procedure consisting of two steps of anion exchange chromatography (DE-52 cellulose) followed by gel filtration as a final step (Sephacryl S-300) was carried out as previously described [13].

3. Results

Our present study was designed to evaluate the effect of several aminoglycosides on RNase P from *D. discoideum* in a cell free system. These included tobramycin, gentamicin, kanamycin, paromomycin and neomycin (Fig. 1). All inhibitors tested are capable of inhibiting the RNase P activity of *D. discoideum* in a dose response manner (Fig. 2A). The concentration of kanamycin, paromomycin, tobramycin, gentamicin and neomycin at which the product formation is reduced by 50% (IC_{50}) is equal to 2.6, 2.2, 1.7, 1.3 and 0.25 mM respectively. When the Mg^{2+} was increased, a reduced inhibition of RNase P cleavage by all aminoglycosides was observed. Increasing Mg^{2+} from 1 to 5 mM, at a constant neomycin concentration, resulted in a considerable recovery of the activity (about 40%) (Fig. 2B). Similar curves were obtained with tobramycin, gentamicin, kanamycin and paromomycin (data not shown). It is obvious that the maximum in-

Table 1

Equilibrium constants derived from primary and secondary kinetic plots (see Fig. 3)

Inhibitor	K_i (μ M)
Neomycin B	143
Gentamicin	734
Tobramycin	1074
Paromomycin	1414
Kanamycin	1871

The K_i values are calculated from the negative intercept of the slope replots (see Fig. 3, inset).

hibitory effect was achieved when the reaction was carried out in the presence of 1 mM Mg^{2+} . At this concentration, the activating site of the enzyme is not saturated with Mg^{2+} [13]. It is important to note that the tested antibiotics exhibit the same effect regardless if the final S-300 step of RNase P purification [13] is included or not.

The inhibition mode of *D. discoideum* RNase P activity by aminoglycosides was further investigated by detailed kinetic analysis in the presence of 1 mM Mg^{2+} . Fig. 3A shows double reciprocal plots with increasing concentration of neomycin which indicate that the inhibition is of a non-competitive type. The slopes of the lines in Fig. 3A were plotted against the concentration of neomycin, and the results are shown in the top panel of Fig. 3A. The linearity of this plot is indicative for simple non-competitive inhibition and permits the graphical determination of $K_i = 143 \mu$ M from the negative intercept of the line with the I-axis. The same kinetic analysis was carried out for tobramycin, gentamicin, kanamycin and paromomycin. All these compounds showed simple non-competi-

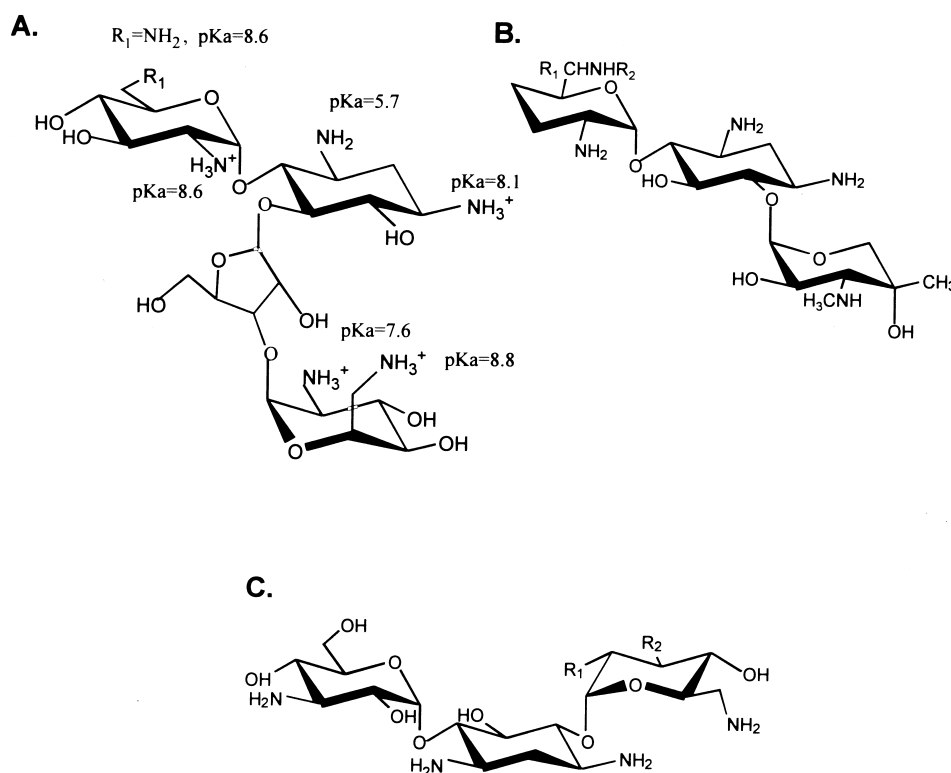


Fig. 1. Structures of aminoglycosides. A: The aminoglycosides neomycin B ($R_1 = NH_2$) and paromomycin ($R_1 = OH$). B: The aminoglycosides gentamicin C₁ ($R_1 = R_2 = CH_3$), gentamicin C₃ ($R_1 = CH_3$, $R_2 = H$), gentamicin C_{1a} ($R_1 = R_2 = H$). C: The aminoglycosides tobramycin ($R_1 = NH_2$, $R_2 = H$) and kanamycin A ($R_1 = R_2 = OH$).

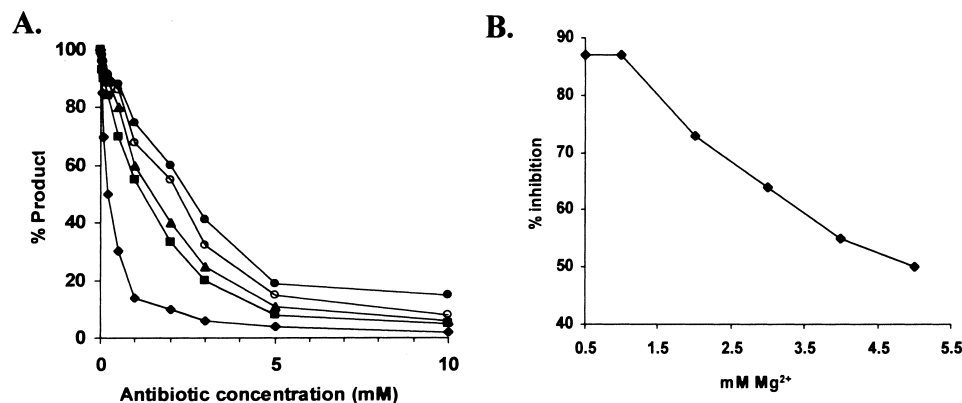


Fig. 2. A: Dose response effects of neomycin (◆), paromomycin (■), gentamicin (▲), kanamycin (○), and tobramycin (●) on the RNase P processing reaction. B: Inhibition of the RNase P reaction in the presence of 0.2 mM neomycin and increasing concentrations of Mg²⁺. All data presented are averaged values of five individual experiments.

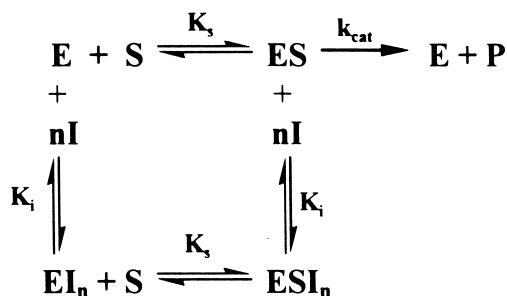
tive inhibition. The corresponding K_i values are shown in Table 1. To evaluate the molecular order of inhibition by aminoglycosides, we used the equation:

$$\frac{v'}{v_0 - v'} = K_i \frac{1}{(I)^n}$$

where n represents the Hill coefficient. This equation was derived from the differential rate law:

$$v' = \frac{V_{\max} S}{(K_s + S) \left(1 + \frac{(I)^n}{K_i} \right)}$$

which corresponds to the kinetic model:



involving non-competitive inhibitor interacting with an enzyme at multiple sites. When $n = 1$, the plot of $v'/(v_0 - v')$ versus $1/[\text{neomycin}]$ is linear. The $v'/(v_0 - v')$ values were calculated at each concentration of pre-tRNA and plotted as a function $1/[\text{neomycin}]$. Such a plot, based on velocities obtained at substrate concentration of 100 nM, is given in Fig. 3B. The linearity of this plot supports that only one molecule of neomycin is kinetically involved in the mechanism of inhibition. Very similar plots were obtained for paromomycin, gentamicin, kanamycin and tobramycin (not shown).

Further kinetic analysis revealed that neomycin competes with Mg²⁺ for common binding sites on the enzyme. Double reciprocal plots $1/v$ versus $1/[\text{Mg}^{2+}]$, with increasing concentration of neomycin in the presence of 100 nM precursor tRNA, indicate that aminoglycosides and Mg²⁺ compete for the same binding sites of the enzyme (Fig. 4A). Furthermore,

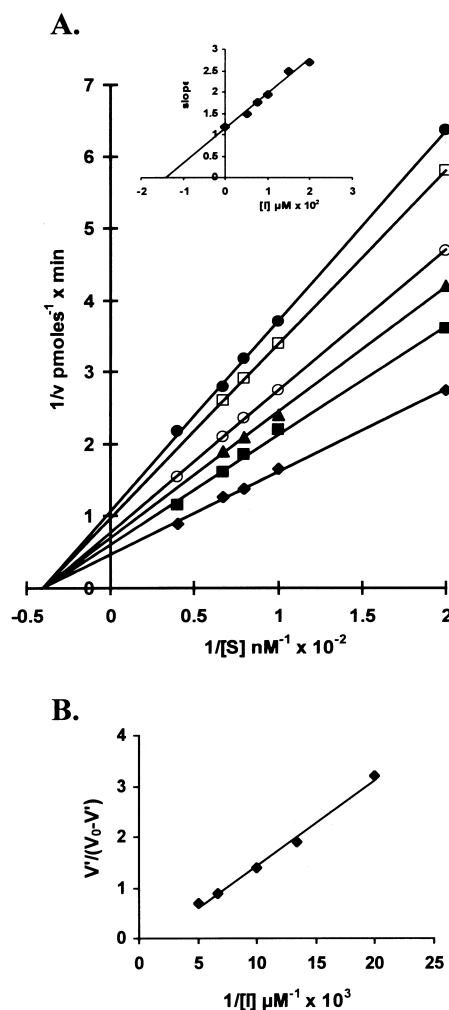


Fig. 3. A: Double reciprocal plot $1/v$ versus $1/[\text{pre-tRNA}]$ for the RNase P reaction in the presence of neomycin. The reaction was carried out at the indicated substrate concentrations in the presence or in the absence of inhibitor. All reactions were carried out in 20 μl buffer D with 1 mM MgCl₂. (◆) Control without inhibitor; with neomycin at 50 μM (■), 75 μM (▲), 100 μM (○), 150 μM (□), 200 μM (●). Inset: Replot of the slopes of the double reciprocal lines versus inhibitor (I) concentration. B: Variation of $v'/(v_0 - v')$ as a function of $1/[I]$. The v' and v_0 values (at 100 nM substrate concentration) were deduced from the double reciprocal plot.

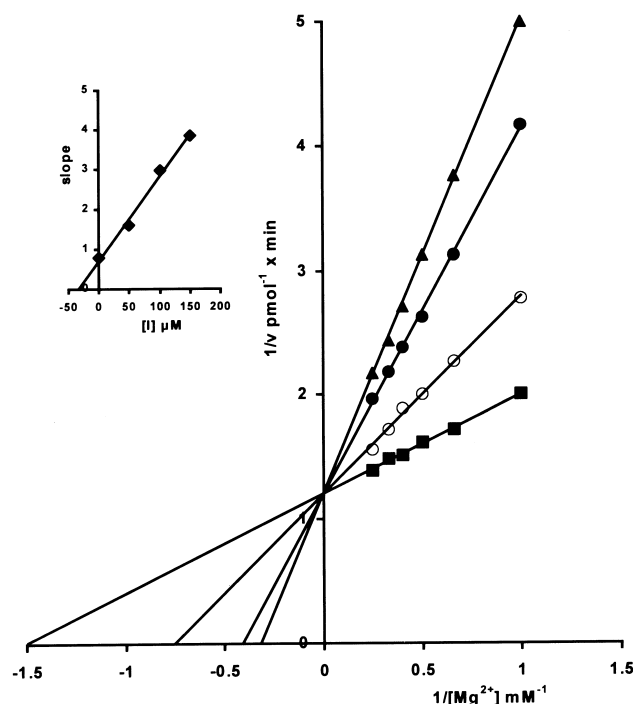


Fig. 4. Double reciprocal plot $1/v$ versus $1/[\text{Mg}^{2+}]$ for the RNase P reaction in the presence or in the absence of neomycin. (■) Control without neomycin; with neomycin at (○) 50 μM , (●) 100 μM , (▲) 150 μM . Inset: Replot of the slopes of the double reciprocal lines versus inhibitor (I) concentration.

the linearity of the slope replot is characteristic of simple competitive interaction (Fig. 4A, inset).

Finally, we investigated the pH dependence on neomycin inhibition. At higher pH values a considerable reduction in the inhibition was observed (pH 8.5) (data not shown), suggesting the importance of the presence of protonated amino groups for the inhibitory effect of neomycin.

4. Discussion

In the present study, it has been revealed that the aminoglycosides tobramycin, gentamicin, kanamycin, paromomycin and neomycin exhibit a dose-dependent inhibition effect on tRNA maturation by eukaryotic nuclear RNase P. These aminoglycosides belong to three different groups; the neomycin group (Fig. 1A), the gentamicin group (Fig. 1B) which is a mixture of gentamicin C_1 , C_{1a} and C_3 , and the kanamycin group (Figure 1C). Detailed kinetic analysis showed that the aminoglycosides behave as classical non-competitive inhibitors. Therefore, the potency of these inhibitors could be assigned on the basis of K_i values. Neomycin is 5.13, 7.5, 9.9 and 13.1 times more potent than gentamicin, tobramycin, paromomycin, or kanamycin respectively. The higher potency of neomycin could be attributed to the high number of amino groups. Neomycin has six amino groups, paromomycin, gentamicin and tobramycin have five amino groups and kanamycin has four amino groups (Fig. 1). Taking into account that neomycin is $9.9\times$ and tobramycin is $1.7\times$ more potent inhibitors than paromomycin and kanamycin, respectively (Table 1), it can be concluded that the ammonium group at the R_1 position of the aminoglycoside molecule (Fig. 1) is important

in the process of inhibition. Considerably weaker inhibition at higher pH values indicates that binding of neomycin B to the enzyme depends on the protonation of its amino group. Since *D. discoideum* RNase P is a ribonucleoprotein it is possible that aminoglycosides bind the polyanionic RNA moiety of the enzyme through ionic interactions.

Our data indicate that protonated amino groups of aminoglycosides play a crucial role in the inhibition of cleavage by the protein-rich RNase P enzyme from *D. discoideum*. The inhibition caused by all aminoglycosides tested was sensitive to Mg^{2+} concentration. When the concentration of Mg^{2+} was increased from 1 to 5 mM, a 40% recovery of the activity was observed. Full recovery of the enzyme activity can not be achieved due to the fact that, above 5 mM, Mg^{2+} binds also to the inactivating metal ion binding site [13]. In agreement with these data, the maximum inhibitory effect of aminoglycosides was observed at 1 mM Mg^{2+} , a situation where the activating site of the enzyme was not saturated with Mg^{2+} . Detailed kinetic analysis showed that Mg^{2+} and aminoglycosides compete for common binding sites. To evaluate the molecular order of RNase P inhibition by neomycin, the $v/(v_0 - v)$ values were calculated at different concentrations of pre-tRNA and replotted as a function of $1/[\text{neomycin}]$. Such a plot, obtained at 100 nM pre-tRNA, is given in Fig. 4. The linearity of this plot indicates that only one molecule of neomycin is kinetically involved in the mechanism of inhibition. Linear plots were taken for paromomycin, gentamicin, kanamycin and tobramycin (not shown). Therefore, we can assume that aminoglycosides compete with Mg^{2+} ions for one binding site, the activating site of *D. discoideum* RNase P [13]. Due to the fact that the stoichiometry was deduced by a kinetic curve, rather than from a real measurement of the amount of bound neomycin, we can not exclude the existence of additional neomycin binding sites on RNase P. Our findings are in agreement with previously reported data on aminoglycoside inhibition of HDV [7], hammerhead ribozyme [20,21], self splicing group I intron [22] and *E. coli* RNase P [10]. It has been suggested by these studies that aminoglycoside antibiotics achieve inhibition of ribozyme cleavage by replacement of structurally important magnesium ions with protonated amino groups.

The effect of tobramycin, gentamicin, kanamycin, paromomycin and neomycin on RNase P activity indicates that these compounds, in addition to their inhibitory effect on protein synthesis, exert a direct effect on tRNA biogenesis. It is becoming evident that RNase P is a valuable experimental system for the in vitro study of the biological activity of antibiotics, which may provide a much more differentiated picture of the molecular basis of antibiotic action. *D. discoideum* RNase P seems to be an excellent eukaryotic model system for the study of complex, relatively protein-rich ribonucleoprotein particles, a category of macromolecular complexes to which the eukaryotic ribosome, RNase MRP, the SRP particle and snRNP particles belong. Studies on *D. discoideum* RNase P allow to evaluate toxic side effects of potential antimicrobial inhibitors in eukaryotic organisms.

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References

- [1] Spahn, C.M.T. and Prescott, C.D. (1996) *J. Mol. Med.* 74, 423–439.
- [2] Moazed, D. and Noller, H.F. (1987) *Nature* 327, 389–394.
- [3] Wallis, M.G. and Schroeder, R. (1997) *Prog. Biophys. Mol. Biol.* 67, 141–154.
- [4] Hermann, T. and Westhof, E. (1998) *Curr. Opin. Biotechnol.* 9, 66–73.
- [5] von Ahsen, U., Davies, J. and Schroeder, R. (1991) *Nature* 353, 368–370.
- [6] Stage, T.K., Hertel, K.J. and Uhlenbeck, O.C. (1995) *RNA* 1, 95–101.
- [7] Rogers, J., Chang, A.H., von Ahsen, U., Schroeder, R. and Davies, J. (1996) *J. Mol. Biol.* 259, 916–925.
- [8] Earnshaw, D.J. and Gait, M.J. (1998) *Nucleic Acids Res.* 26, 5551–5561.
- [9] Zapp, M.L., Stern, S. and Green, M.R. (1993) *Cell* 74, 969–978.
- [10] Mikkelsen, N.E., Mathias, B., Virtanan, A. and Kirsebom, L.A. (1999) *Proc. Natl. Acad. Sci. USA* 96, 6155–6160.
- [11] Frank, D.N. and Pace, N.R. (1998) *Annu. Rev. Biochem.* 67, 153–180.
- [12] Guerrier-Takada, C., Haydock, K., Allen, L. and Altman, S. (1986) *Biochemistry* 25, 1509–1515.
- [13] Tekos, A., Stathopoulos, C. and Drinas, D. (1998) *Biochemistry* 37, 15474–15480.
- [14] Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N. and Altman, S. (1983) *Cell* 35, 849–857.
- [15] Pannucci, J.A., Haas, E.S., Hall, Th.A., Harris, J.K. and Brown, J.W. (1999) *Proc. Natl. Acad. Sci. USA* 96, 7803–7808.
- [16] Chamberlain, J.R., Lee, Y., Lane, W.S. and Engelke, D. (1998) *Genes Dev.* 12, 1678–1690.
- [17] Stathopoulos, C., Kalpaxis, D.L. and Drinas, D. (1995) *Eur. J. Biochem.* 228, 976–980.
- [18] Eder, P.S., Kekuda, R., Stolc, V. and Altman, S. (1997) *Proc. Natl. Acad. Sci. USA* 94, 1101–1106.
- [19] Papadimou, E., Georgiou, S., Tsambaos, D. and Drinas, D. (1998) *J. Biol. Chem.* 273, 24375–24378.
- [20] Clouet-d’Orval, B., Stage, T.K. and Uhlenbeck, O.C. (1995) *Biochemistry* 34, 11186–11190.
- [21] Hermann, T. and Westhof, E. (1998) *J. Mol. Biol.* 276, 903–912.
- [22] Hoch, I., Berens, C., Westhof, E. and Schroeder, R. (1998) *J. Mol. Biol.* 282, 557–569.