Prevention of passively transferred experimental autoimmune myasthenia gravis by an in vitro selected RNA aptamer

Byounghoon Hwang[a], Kyungsook Han[b], Seong-Wook Lee[a],*

[a]*Department of Molecular Biology, Dankook University, Seoul 140-714, South Korea*
[b]*School of Computer Science and Engineering, Inha University, Incheon 402-751, South Korea*

Abstract **Myasthenia gravis (MG) and its animal model, experimental autoimmune MG (EAMG), are mainly caused by autoantibodies directed against acetylcholine receptors (AChR) located in the postsynaptic muscle membrane. Previously, we isolated an RNA aptamer with 2′-fluoropyrimidines using in vitro selection techniques that acted as an effective decoy against both a rat monoclonal antibody called mAb198, which recognizes the main immunogenic region on the AChR, and a significant fraction of patient autoantibodies with MG. To investigate the therapeutic potential of the RNA, we tested the ability of the RNA aptamer to protect the receptors in vivo from mAb198. Clinical symptoms of EAMG in rats engendered by passive transfer of mAb198 were efficiently inhibited by a truncated RNA aptamer that was modified with polyethylene glycol, but not by control scrambled RNA. Moreover, the loss of AChR in the animals induced by the antibody was also significantly blocked with the modified RNA aptamer. These results suggested that RNA aptamers could be applied for antigen-specific treatment for autoimmune diseases including MG.**
© 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Experimental autoimmune myasthenia gravis; In vitro selection; RNA aptamer; Acetylcholine receptor; Autoantibody

1. Introduction

Myasthenia gravis (MG) is a neuromuscular disorder associated with muscular weakness and fatigability. The pathogenesis of MG mainly results from antibody-mediated autoimmune response to nicotinic acetylcholine receptors (AChR) located in the postsynaptic muscle cell membrane [1]. Experimental autoimmune myasthenia gravis (EAMG) can be generated in animals by active immunization with purified AChR or passive transfer of anti-AChR antibodies [2]. The majority of anti-AChR antibodies found in both human patients with MG and rats with EAMG bind to the main immunogenic region (MIR) on the extracellular domain of the AChR α-subunit [3,4]. Such autoantibodies cause internalization and degradation of the AChR available on the skeletal muscle at neuromuscular junctions, resulting in the failure of neurotransmission and paralysis [1,2]. Because the current MG therapies including general immunosuppression often cause

toxic side effects [1], development of agents that can more specifically inhibit MG is warranted.

Since the pathogenesis of MG is mainly due to autoantibodies to AChR, much effort has been directed toward the development of small molecules that block interaction of the antibodies with the receptors [5–7]. Short RNA aptamers that have been isolated to a wide range of proteins with iterative in vitro selection techniques from a combinatorial RNA library [8,9] have been reported to act as specific decoys and inhibit the physiological functions known to be associated with the target proteins in cells and in animal models [10]. The rationale of these experiments is that a short RNA molecule with certain sequence can form a fairly stable and unique tertiary structure via intramolecular base-pairing [11]. Noticeably, attachment of a polyethylene glycol (PEG) to the 5′ end of an aptamer has been shown to enhance in vivo bioavailability of the aptamer [12,13]. Thus, RNA molecules could be alternative candidates for specific and effective inhibitors of MG autoantibodies.

Previously, we employed in vitro selection methods and successfully isolated a short and nuclease-resistant RNA aptamer with 2′-amino- [14] or 2′-fluoro-modified pyrimidines [15] that can bind both a rat monoclonal antibody, called mAb198, that is specific to the MIR on human AChR, and patient autoantibodies with MG with high specificity and high affinity. Surprisingly, the sequence of the RNA aptamer with 2′-fluoropyrimidines was the same as that of RNA with 2′-aminopyrimidines [15]. These RNA aptamers have been shown to act as decoys to protect AChR from the effects of the antibodies in human cells. Moreover, we could improve binding activity and bioactivity of the RNA aptamer with 2′-amino by extended sequence selection [16]. In this study, we present the ability of the RNA aptamer to inhibit EAMG in animal models. We demonstrated that the selected RNA aptamer with 2′-fluoropyrimidines that was truncated and modified by attachment of PEG at its 5′ end showed no significant differences in binding affinity to mAb198 and bioactivities protecting AChR on human cells from both mAb198 and patient autoantibodies, compared to the unmodified 2′-fluoro RNA aptamer. Moreover, the modified aptamer protected clinical symptoms of EAMG in animals passively transferred by mAb198 and blocked the antibody-mediated loss of muscle AChR in the animals.

2. Materials and methods

2.1. Antibodies, AChR, and cells
Rat anti-MIR mAb198 and rat hybridoma cell lines producing

mAb198, kindly provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA, USA), were prepared, and characterized as described [6]. Serum samples from a patient with MG (batch #2051) were purchased from The Binding Site (San Diego, CA, USA). According to the manufacturer, the radioimmunoassay titer of anti-human AChR in the patient's serum was very high. The major extracellular domain of the human AChR α-subunit (amino acids 1–210) was a gift from Sohel Talib (Gencell, Santa Clara, CA, USA). The TE671 human medulloblastoma cell line (ATCC 8805-CRL) of rhabdomyosarcoma origin [17] expresses receptors for acetylcholine apparently identical to human AChR [18] and was used in AChR down-modulation experiments.

2.2. Preparation and analysis of selected RNA aptamer

Full-length RNA aptamer with 2′-deoxy-2′-fluoropyrimidines (Ambion) previously selected against mAb198 or its truncated form (fMG RNA or tMG RNA, respectively) was synthesized by in vitro transcription of synthetic DNA templates, radiolabeled, and isolated as described [15,19]. The sequence of fMG RNA was 5′-GGGAGAG-CGGAAGCGUGCU*GGGCCGGAGGUUAGCUUGCCCAUGGCA-AGCAGGGCGCCACGGACC*CAUAACCCAGAGGUCGAUGGA-CAUAACCCAGAGGUCGAUGGAUCCC-3′, where the underlined sequence represents selected nucleotides (nt) and the 46-nt-long truncated sequence in italics is a minimal binding domain. Truncated RNA aptamer was coupled to 40-kDa PEG to its 5′ end (Peg-tMG RNA) to increase its residence time in animal plasma as described [20]. For binding affinity assays, competition binding was performed by incubating 100 pM of ^{32}P-labeled fSE RNA, 50 nM mAb198, and excess amount of unlabeled competitor RNA in 100 μl binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, 2 mM dithiothreitol, and 1% bovine serum albumin) for 30 min at room temperature with shaking. Antibody–RNA complexes were immunoprecipitated with 20 μl of protein G-Sepharose beads (Pharmacia Biotech), and bound RNAs eluted from the pellets and analyzed on a 6% polyacrylamide gel with urea. The percentage of RNA bound to mAb198 was calculated as the fraction of radioactivity present in the antibody–RNA complexes in the absence of any competitors. The K_d of the competitor RNA was estimated to be equivalent to its 50% inhibitory concentration as described [21].

2.3. Assay of AChR down-modulation

AChR down-modulation was monitored as described [14–16]. MAb198 (5 nM) or autoantibodies from a patient with MG were preincubated in 90 μl of Dulbecco's modified Eagle's medium containing 10 mM MgCl$_2$, 1% bovine serum albumin, and 10% fetal bovine serum with tested RNA for 30 min at room temperature. These mixtures were then added to TE671 cells in the presence of 40 μg/ml of cycloheximide and incubated at 37°C for 4 h. Next, 10 μl of medium with 50 nM of ^{125}I-radiolabeled α-bungarotoxin (α-Bgt) was added and incubated for 3 h more. The cells were then washed, and the bound α-Bgt was quantitated using a γ 5000 counter (Packard Instruments). Incubation of TE671 cells with 5 nM of mAb198 by itself reproducibly reduced AChR expression of the cell surface by approximately 50%. This level of antigenic down-modulation was considered 100%, and other values were normalized to this amount. For antigenic down-modulation experiments with patient autoantibodies, patient sera were added to TE671 cells at concentrations determined to engender similar levels of antigenic down-modulation as 5 nM of mAb198: 1 μl of 1:2 dilution of patient sera (data not shown).

2.4. Prevention of EAMG in rats

Six-week-old female Lewis rats weighing 100–150 g were used for i.p. passive transfer of 100 μg of mAb198 in phosphate-buffered saline (PBS). The effect of RNA aptamer was analyzed by injecting a mixture of mAb198 and RNA aptamer after preincubation for 1 h at room temperature. To ensure that intact RNA aptamer was continuously present in the circulation, the aptamer was injected two more times. There was no significant inhibition of EAMG with only initial single input of the RNA. Twenty-four hours after mAb198 injection the rats were assessed for EAMG symptoms and scored according to the following grade: 0, normal; 1, weak grasp after a few trials; 2, incomplete paralysis of hind limbs; 3, severe paralysis and moribund; and 4, death. Rats with intermediate symptoms were scored with intermediate scales. At the end of the experiment, the rats were killed

and the AChR content in the extract of hind limb muscles of each rat was monitored by radioimmunoassay as described [22]. The values were expressed as a percentage of the AChR amount in rats injected only with PBS.

3. Results and discussion

3.1. Binding affinity of RNA aptamer to mAb198

Previously, we isolated an 89-nt-long nuclease-resistant RNA aptamer, termed fMG RNA, which bound to mAb198 very avidly ($K_d \approx 25$ nM) and specifically, from a large ($\sim 10^{14}$ molecules) combinatorial RNA library with every pyrimidine modified at its 2′ position by a fluoro group [15]. For the scale of efficient synthesis necessary for in vivo applications, however, fMG RNA must be shortened to at most 50 nt. A 46-nt-long truncated version of fMG RNA, termed tMG RNA, was observed to contain the most critical sequences for mAb198 binding from deletion mapping analysis (Fig. 1A, data not shown). Scrambling the five nucleotides that form the internal loop of tMG RNA disrupted the aptamer binding to mAb198 (Fig. 1B), hence this RNA (Scramble RNA) was used as a negative control for tMG RNA. For the assessment of in vivo activity of the aptamer, Peg-tMG RNA was generated because its conjugation to an RNA ap-
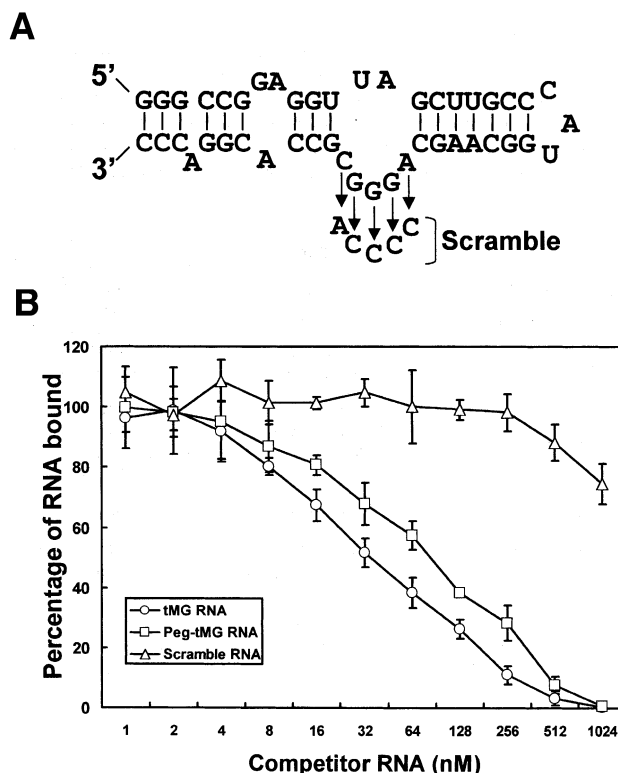

Fig. 1. Sequence, structure, and binding affinity of RNA aptamer isolated against mAb198. A: Predicted secondary structure of tMG RNA, a 46-nt truncate of a full-length RNA aptamer (fMG RNA) to mAb198, was determined using the MULFOLD program [27]. Mutation of five nucleotides in tMG RNA yields an inactive aptamer, Scramble RNA. B: The percentage of fMG RNA bound to mAb198, normalized to binding in the absence of any competitor RNA, was plotted as a function of each competitor RNA (tMG RNA, Peg-tMG RNA, or Scramble RNA). Values shown are averages of measurements performed in triplicate, and bars indicate standard errors.

tamer with small size has been shown to reduce rapid tissue diffusion and renal clearance of the aptamer [12,13].

To determine the binding affinity of the truncated aptamers, a competition binding assay was carried out by incubation of trace amounts of radiolabeled fMG RNA and increasing amounts of unlabeled competitor RNA (Fig. 1B). An equilibrium dissociation constant (K_d) of 30 nM, an affinity that was very similar to that of fMG RNA (K_d of 25 nM), was detected for tMG RNA. Moreover, the binding affinity of Peg-tMG RNA (K_d of 75 nM) to mAb198 was only about three-fold less than that of fMG RNA. Thus, both a truncated version of the selected RNA aptamer against mAb198 and its PEG-conjugated form retain high affinity of the aptamer to the antibody. By contrast, Scramble RNA could hardly displace the binding of fMG RNA to mAb198 even at the highest concentration of RNA, indicating that mutation of the internal loop regions disrupts the aptamer's binding activity to the antibody.

3.2. Inhibition of autoantibody-mediated antigenic modulation of AChR expression by RNA aptamer

Once it was ascertained that the truncated and modified RNA aptamer with PEG bound to mAb198 with high affinity, we next determined if the RNA could protect AChR on human cells from the effects of mAb198. Incubation of human TE671 cells with mAb198 resulted in a dose- and time-dependent degradation of AChR on the cell surface (data not shown) [23], which is referred to as antigenic down-modulation. To assess the modified aptamer's ability to inhibit antigenic down-modulation induced by the antibody, TE671 cells were incubated with mAb198 in the presence or absence of the RNAs (Fig. 2A). AChR degradation was assessed by measuring the amount of [^{125}I]α-Bgt that could bind the AChRs remaining on the cells after treatment with the antibody, as previously described [14]. While a negative control RNA competitor, such as Scramble RNA, showed little inhibition of the mAb198-mediated AChR down-modulation, fMG RNA showed efficient protection of TE671 cells from the antibody and inhibited AChR down-modulation by up to 65% as previously shown [15]. Moreover, both tMG RNA and Peg-tMG RNA also efficiently blocked TE671 cells from mAb198 and inhibited antigenic down-modulation by up to 62% and 50%, respectively. Furthermore, inhibition of AChR down-modulation with the aptamers was dose-dependent (data not shown). Thus, truncation and PEG conjugation of the RNA aptamer selected against mAb198 showed no significant difference in the protection of cells from the antibody, compared with the originally selected fMG RNA.

We have previously observed that fMG RNA could cross-react with anti-AChR autoantibodies from MG patient sera. This result was probably due to the fact that most pathologically relevant autoantibodies from MG patients appear to bind the AChR's MIR that was recognized by mAb198 [2,24]. However, the RNA blocked a certain fraction of the antibodies in the MG patient sera from binding to, and hence the down-modulation of, AChR on TE671 cells with patient variability [15]. This is probably due to different immune responses against AChR in the different individuals. Nevertheless, fMG RNA could inhibit the autoantibodies at least in one patient in a dose-dependent manner almost as well as it could protect cells from mAb198. Thus, we wanted to determine if the modified RNA aptamer could inhibit the down-
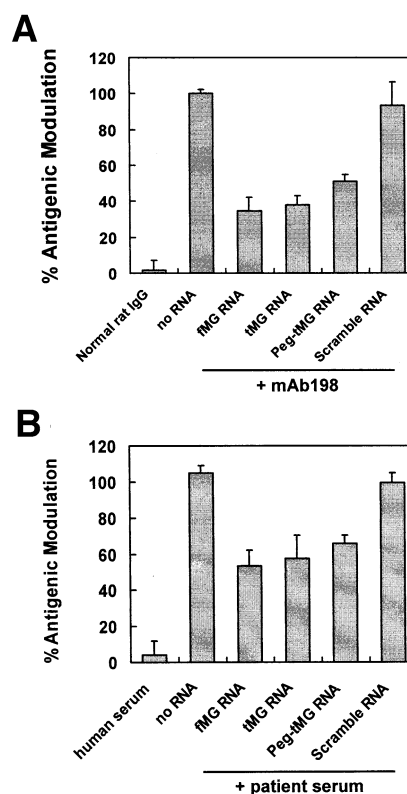

Fig. 2. Inhibition of antigenic down-modulation mediated by either mAb198 (A) or patient serum with MG (B) in cells with RNA aptamers. A: TE671 cells were incubated with 5 nM of normal IgG, 5 nM of mAb198 alone, or mAb198 (5 nM) preincubated with 5 μM of each RNA aptamer (fMG RNA, tMG RNA, Peg-tMG RNA, or Scramble RNA). Down-modulation of AChR expression on the cell surface was analyzed by [^{125}I]α-Bgt binding. Values were expressed as a percentage of AChR down-modulation engendered by mAb198 alone (5 nM). Values shown represent the means ± S.D. of three separate determinations. B: TE671 cells were incubated with 1 μl normal human serum, or 1 μl of 1:2 dilution of MG patient serum either alone or after preincubation with 5 μM of each RNA aptamer. AChR down-modulation was measured as described.

modulation of AChR expression mediated by autoantibodies in the patient sera (Fig. 2B). Addition of fMG RNA, but not control RNA such as Scramble RNA, protected a significant fraction of AChR on TE671 cells from the patient's autoantibodies, and inhibited the AChR down-modulation by 47%. Truncated tMG RNA and Peg-tMG RNA also efficiently protected a certain fraction of the cells from the autoantibodies, and inhibited the antigenic down-modulation by 43% and 35%, respectively. Thus, truncation and PEG conjugation of the RNA aptamer against mAb198 did not severely affect the aptamer's ability to block AChR down-modulation mediated by the most inhibited patient sera.

3.3. Inhibition of mAb198-mediated passive transfer of EAMG in rats by RNA aptamer

Once the truncated and modified RNA aptamer with PEG was shown to interact with mAb198 with high affinity and inhibited AChR degradation induced by autoantibodies in cells, we examined whether the modified RNA aptamer could block EAMG generated by mAb198 to evaluate the therapeutic potential of the RNA aptamer (Fig. 3). Severe muscular weakness was observed in 6-week-old rats within 24 h after a
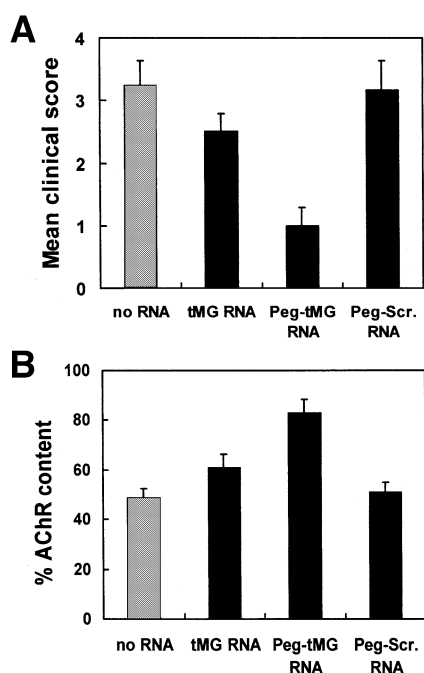
Fig. 3. Protection from EAMG induced by passive transfer of mAb198 with RNA aptamer. Lewis rats were injected i.p. with 0.1 mg of mAb198 that had been preincubated in the absence or presence of 1 mg of each RNA aptamer (tMG RNA, Peg-tMG RNA, or Peg-Scr. RNA, which was a modified Scramble RNA with PEG). A: The clinical symptoms were scored as described in Section 2. B: Muscle AChR content in hind limbs of the treated animals was assessed as described. The results are the means ± S.E.M. of three different animals in two independent sets of experiments.

single i.p. injection of 0.1 mg of mAb198 (Fig. 3A). However, this progression of clinical symptoms induced by the antibody was greatly reduced by repeated injection of Peg-tMG RNA. By contrast, non-PEG-conjugated tMG RNA showed only mild abolition of EAMG even with repeated treatment, indicating that the increase in the circulation residence of the RNA aptamer in animals by PEG conjugation was truly important for the improvement of in vivo bioavailability of the aptamer. Noticeably, PEG-conjugated Scramble RNA (Peg-Scr. RNA) hardly prevented EAMG signs induced by mAb198. Thus, the protective effect of Peg-tMG RNA against EAMG in animals is not due to the non-specific effect that PEG-conjugated RNA from itself might have, but due to the specific binding of the RNA aptamer with mAb198. Animals injected only with the modified RNA aptamer showed no signs of EAMG (data not shown). To confirm the inhibition of EAMG by Peg-tMG RNA, we measured the muscle concentration of AChR in the hind limb by radioimmunoassay at 24 h following injection of mAb198 with or without RNA aptamers (Fig. 3B). Control animals injected with mAb198 alone lost almost 51% of their muscular AChR content, as reported, whereas animals treated with Peg-tMG RNA and mAb198 showed a significantly lower loss of the AChR (approximately 17% AChR loss). On the other hand, tMG RNA showed only partial protection from AChR loss induced by mAb198 (39% AChR loss), compared with Peg-tMG RNA, confirming the enhancement of in vivo bioavailability of the RNA aptamer by PEG conjugation. Animals injected with Peg-Scr. RNA and mAb198 did not show any protection from AChR loss (49% AChR loss), compared with animals

with the antibody alone, confirming the specific protection of animal neuromuscles from AChR loss induced by the antibody with the Peg-tMG RNA aptamer. Thus, it was concluded that Peg-tMG RNA could effectively inhibit mAb198-mediated passive transfer of EAMG in animals.

The pathogenic occurrence of autoimmune responses in MG patients could be potentially modulated with small molecules that bind the combining site of autoantibodies, and hence inhibit their interaction with the MIR of the autoantigen, AChR. Thus, MIR mimotopes, such as short peptides against autoantibody, or anti-AChR Fab fragments have been developed as protectors of cells from the autoantibodies [5–7]. However, difficulties in isolating short peptide binding target proteins with high affinity [25] or possible AChR loss by the Fab fragment in MG patients [6] might limit their clinical applicability. As an alternative anti-MG therapeutic candidate, we have previously isolated a specific RNA aptamer with high avidity against anti-AChR antibody. In this study, we observed that the RNA aptamer could efficiently protect animals from EAMG passively induced by the antibody. For the enhancement of in vivo bioavailability of the RNA aptamer, we truncated and modified the RNA with PEG. This modified RNA aptamer retained both high binding affinity to the antibody and effective bioactivity blocking AChR on human cells from the autoantibodies. Importantly, the modified RNA aptamer alone neither induced EAMG nor showed any loss of AChR in animals. Moreover, short oligonucleotides such as RNA aptamers are intrinsically poor antigens, and they are appropriate for chemical synthesis at a large-scale. These results combined with previous observations [14–16], therefore, strongly suggest that RNA aptamer against anti-AChR antibody could be an attractive lead compound for the development of specific and non-toxic anti-MG drugs. However, heterogeneous populations of anti-AChR autoantibodies present in MG patient sera [26] will probably limit the clinical utility of the RNA. Indeed, the single kind of RNA aptamer against only mAb198 cross-reacted poorly with other anti-MIR antibodies, inhibited bioactivity of autoantibodies only from a limited number of patients at a certain level, [14,15] and hardly protected EAMG actively induced by immunization with AChR in animals (data not shown). Therefore, to be therapeutically valuable, it should be worthwhile to isolate RNA aptamers against additional anti-MIR antibodies, or a pool of various autoantibodies in MG patients. If a combination of such RNAs prevents the binding of a wider variety of the anti-AChR autoantibodies in MG patients, the RNAs can then be applied to specific agents against MG.

References

[1] Drachman, D.B. (1994) New Engl. J. Med. 330, 1797–1810.
[2] Lindstrom, J., Shelton, D. and Fugii, Y. (1988) Adv. Immunol. 42, 233–284.
[3] Tzartos, S.J., Rand, D.E., Einarson, B.L. and Lindstrom, J.M. (1981) J. Biol. Chem. 256, 8635–8645.
[4] Tzartos, S.J., Seybold, M.E. and Lindstrom, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 188–192.

[5] Araga, S., Galin, F.S., Kishimoto, M., Adachi, A. and Blalock, J.E. (1996) J. Immunol. 157, 386–392.

[6] Papanastasiou, D., Poulas, K., Kokla, A. and Tzartos, S.J. (2000) J. Neuroimmunol. 104, 14–132.

[7] Venkatesh, N., Im, S.-H., Balass, M., Fuchs, S. and Katchalski-Katzir, E. (2000) Proc. Natl. Acad. Sci. USA 97, 761–766.

[8] Tuerk, C. and Gold, L. (1990) Science 249, 505–510.

[9] Elligton, A.D. and Szostak, J.W. (1990) Nature 346, 818–822.

[10] Sullenger, B.A. and Gilboa, E. (2002) Nature 418, 252–258.

[11] Gold, L., Allen, P., Binkley, J., Brown, D., Schneider, D., Eddy, S. R., Tuerk, C., Green, L., Macdougal, S. and Tasset, D. (1993) in: The RNA World (Gestelend, R.F. and Atkins, J.F. Eds.), pp. 497–510, Cold Spring Harbor Press, Cold Spring Harbor, NY.

[12] Tucker, C.E., Chen, L.S., Judkins, M.B., Farmer, J.A., Gill, S.C. and Drolet, D.W. (1999) J. Chromatogr. B Biomed. Sci. Appl. 732, 203–212.

[13] Watson, S.R., Chang, Y.F., O'Connell, D., Weigand, L., Ringquist, S. and Parma, D.H. (2000) Antisense Nucleic Acid Drug Dev. 10, 63–75.

[14] Lee, S.-W. and Sullenger, B. (1997) Nat. Biotechnol. 15, 41–45.

[15] Seo, H.-S. and Lee, S.-W. (2000) J. Microbiol. Biotechnol. 10, 707–713.

[16] Hwang, B. and Lee, S.-W. (2002) Biochem. Biophys. Res. Commun. 290, 656–662.

[17] Stratton, M.R., Darling, J., Pikington, G.J., Lantos, P.L., Reeves, B.R. and Cooper, C.S. (1989) Carcinogenesis 10, 899–905.

[18] Luther, M.A., Schoepfer, R., Whiting, P., Casey, B., Blatt, Y., Montal, M.S., Montal, M. and Lindstrom, J. (1989) J. Neurosci. 9, 1082–1096.

[19] Fizwater, T. and Polisky, B. (1996) Methods Enzymol. 267, 275–301.

[20] Ostendorf, T., Kunter, U., Eitner, F., Loos, A., Regele, H., Kerjaschki, D., Henninger, D.D., Janjic, N. and Floege, J. (1999) J. Clin. Invest. 104, 913–923.

[21] White, R., Rusconi, C., Scardino, E., Wolberg, A., Lawson, J., Hoffman, M. and Sullenger, B. (2001) Mol. Ther. 4, 567–573.

[22] Souroujon, M.C., Pizzighella, S., Mochly-Rosen, D. and Fuchs, S. (1985) J. Neuroimmunol. 8, 159–166.

[23] Sophianos, D. and Tzartos, S. (1989) J. Autoimmun. 2, 777–789.

[24] Saedi, M.S., Anand, R., Conroy, W.G. and Lindstrom, J.M. (1990) FEBS Lett. 267, 55–59.

[25] Gold, L., Polisky, B., Uhlenbeck, O.C. and Yarus, M. (1995) Annu. Rev. Biochem. 64, 763–795.

[26] Vincent, A., Whiting, P.J., Schluep, M., Heidenreich, F., Lang, B., Roberts, A., Willcox, N. and Newson-Davis, J. (1987) Ann. NY Acad. Sci. 505, 106–120.

[27] Jaeger, J., Turner, D. and Zuker, M. (1989) Proc. Natl. Acad. Sci. USA 86, 7706–7710.