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Towards artificial ribonucleases: the sequence-specific cleavage of RNA in a duplex

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ABSTRACT

Lanthanide complexes covalently attached to oligonucleotides have been shown to cleave RNA in a sequence-specific manner. Efficient cleavage, however, is at present limited to single-stranded RNA regions, as RNA in a duplex is considerably more resistant to strand scission. To overcome this limitation, we have designed and synthesised artificial nucleases comprising lanthanide complexes covalently linked to oligodeoxyribonucleotides which cleave a partially complementary RNA at a bulged site, in the duplex region. Strand scission occurs at or near the bulge. Cleavage of the RNA target by the metal complex can be addressed *via* the major or the minor groove. In an example of a competitive situation, where the cleavage moiety has access to both a bulge and a single-strand region, transesterification at the bulge is favoured. Such artificial ribonucleases may find application as antisense agents and as tools in molecular biology. In addition, the results may have importance for the design of artificial ribonucleases which are able to act with catalytic turnover.

INTRODUCTION

Several groups have recently reported the sequence-specific hydrolytic cleavage of ribonucleic acid (RNA) using metal complexes covalently linked to oligonucleotides (1–5). This is of current interest in the antisense field (6–9) because it may permit the selective destruction of a targeted messenger RNA *in vivo* without the need for cellular enzymes or endogenous cofactors. Highly efficient RNA cleavage has been shown with macrocyclic lanthanide complexes (2,4,5), which are among the most potent transphosphorylation catalysts known and also possess favourable properties for eventual *in vivo* applications. In both cases, cleavage of the target was confined to the single-stranded region, a few nucleotides from the end of the duplex formed by the conjugate and the target RNA. Furthermore, an excess of cleaver conjugate was used to achieve efficient cleavage. The potency of such artificial ribonucleases might be raised if the cleavage process could be extended into the duplex region. This would not only increase the number of possible cleavage sites but, more importantly, offer the possibility of catalytic turnover (10). A fundamental requirement of catalysis is a rapid dissociation of the two RNA fragments from the nuclease after the cleavage process in order to avoid product inhibition. Hence, whereas formation of a stable duplex between the nuclease and the target is required in a first step, the final complex between the nuclease and the RNA fragments after the reaction should be sufficiently destabilised to allow rapid nuclease release (11). This condition is not fulfilled if the target is cleaved in the single strand (Fig. 1A) but it could arise if cleavage occurred within the duplex (Fig. 1B). On the other hand, it has been reported that double-stranded RNA is considerably more resistant to metal ion promoted transesterification than its single stranded counterpart (12) rendering cleavage of RNA within a duplex difficult or impossible. In the course of our research directed towards the sequence specific chemical cleavage of RNA, however, we found that bulged RNA residues are—in contrast to paired residues—susceptible to metal promoted transesterification (Hüsken,D., Goodall,G., Blommers,M.J.J., Jahnke,W., Hall,J., Häner,R., Moser,H.E. manuscript in preparation). Consequently, we applied this finding to our work with oligonucleotide lanthanide conjugates. Here, we report the efficient sequence-specific cleavage of a synthetic RNA in the duplex region by use of oligonucleotides bearing lanthanide complexes. RNA cleavage is made possible by the enforced presence of a cleavage-susceptible bulge in the RNA target strand (13).

RESULTS AND DISCUSSION

The oligonucleotide metal complex conjugates used in this study were prepared from lanthanide complexes **1–3** (Fig. 2) in analogy to the published procedure (4). Amino groups were introduced into oligonucleotides **4–7** using the appropriately modified building blocks (14–16) for conjugates **8–12** (henceforth referred to as endoconjugates) or the commercially available linker for conjugate **13** (referred to as exoconjugate). The lanthanide complexes **1–3** were covalently linked to the amino oligonucleotides *via* their respective *N*-hydroxysuccinimide ester or isothiocyanate derivatives. All amino oligonucleotides and conjugates were purified by reverse phase HPLC and were characterised by matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS), and capillary gel electrophoresis (CGE) or polyacrylamide gel electrophoresis (PAGE).

In a first experiment using endocleaver **8**, the reaction with a fully complementary 29mer RNA target (**14**) was compared to that with a similar 31mer target (**15**), identical to the former

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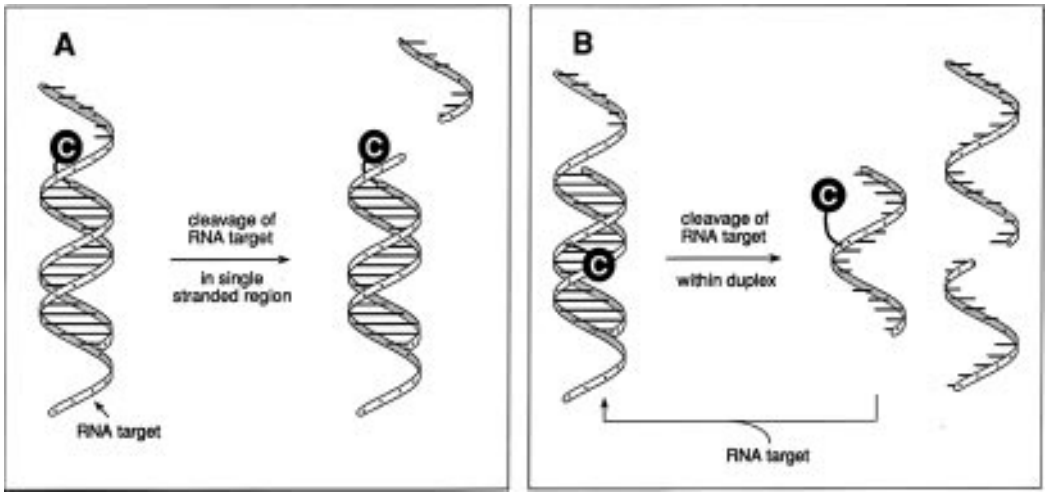

Figure 1. Schematic representation of cleavage of RNA by an artificial ribonuclease; (**A**) cleavage in the single-stranded region, and (**B**) in the duplex formed by the artificial nuclease and the target.

except for two additional nucleotides in the middle of the sequence, which are bulged out upon hybridisation with the conjugate. The two target oligoribonucleotides were incubated for 16 h at 37°C (pH 7.4) with an excess of conjugate **8** and the products were analysed on a 12% denaturing polyacrylamide gel. The autoradiograph depicted in Figure 3A shows that the fully matched target RNA **14** was only cleaved slightly (Fig. 3A, lane 5 and illustration, Fig. 3B) at a single site just opposite the nucleotide carrying the metal complex. On the other hand, the target RNA **15** (Fig. 3A, lane 13) was cleaved almost quantitatively under identical conditions. Cleavage is not restricted to the bulge site but is also observed at the phosphodiesters adjacent to the bulge (Fig. 3B). Minor cleavage occurred—as in the case of the perfect duplex—at the GA-site opposite the metal complex-carrying thymidine. Quantitative evaluation of the reaction products reveals how much more efficient cleavage is at the double bulge than in the duplex (92 versus 7% respectively). The control reactions show that no RNA cleavage is effected by the amino oligonucleotide **4** alone (lanes 7 and 15), the free metal complex 1 (lanes 8 and 16) or a mixture of amino oligonucleotide and free metal complex together (lanes 6 and 14)—neither in the duplex nor at the bulge. Furthermore, a DNA target with an identical sequence to that of an RNA, i.e. forming a bulge at the same site, was not cleaved to any observable extent (data not shown).

Having established an enhanced susceptibility to cleavage of unpaired ribonucleotides in the duplex region, the effects of the attachment of the metal complex to other topological sites of the oligonucleotide conjugate were investigated. Thus, the RNA target **15** was treated with endoconjugates **8–12**. Whereas the metal complexes of conjugates **8–11** (lanes 5–8) were designed to reach the bulged nucleotide across the minor groove, that of conjugate **12** (lane 9) has to act through the major groove. The results are shown and illustrated in Figure 4. The autoradiograph depicted in Figure 4 shows that the conjugate **8** is the most potent compound of the series, cleaving 90% of the target in 16 h at 37°C. The cleavage activity of **8** can directly be compared with that of conjugates **9** and **10**, since in all three compounds the metal complex is attached to the oligonucleotide via the ribosyl 2′-position. In agreement with previous findings (4), complexes of europium are found to be superior to lanthanum (cf. **8** versus **9**; 90 and 13% cleavage, respectively). Conjugate **8** also shows better activity than **10** (23% of target cleaved), which differs from the former with regard to the site of attachment of the metal complex and to the two methyl substituents at the hydrazone carbons (17). Conjugate **11**, in which the complex is linked to the oligonucleotide via a carbanucleotide, is almost as active as **8**, cleaving 61% of the target RNA. This is not surprising in view of the relatively small topological difference between the sites of attachment of the two metal complexes. Finally, conjugate **12** (21% of target cleaved), in which the complex is linked to the oligonucleotide via the thymidine 5-position and which reaches the bulge through the major groove, has an activity similar to those of **9** and **10**. The lower activity of **12** compared to **8** and **11** may reflect a less suitable linker, or a more difficult approach to the site of reaction. As no efforts were made to optimise the linker nor the site of attachment for this line of attack, it is not possible to draw firm conclusions concerning this point.

One aspect of great interest is the comparative cleavage reactivities of single-stranded RNA and the bulge site. This issue was addressed by comparing the behaviour of exoconjugate **13** with target oligoribonucleotides **16**, containing a double bulge four base pairs from the end of the duplex, and **17**, a perfectly complementary target. Both of these RNAs possess single-stranded regions adjacent to the duplex region. However, in the former case the metal complex has the possibility to cleave two different target sites, at the bulged nucleotides in the duplex, or in the single-stranded region. The results are shown in Figure 5. In target **17**, having no bulge (lane 12), cleavage occurs almost exclusively in the single-stranded region. In contrast, only bands corresponding to cleavage in the bulge region are observed with the target **16**. It cannot be excluded from these experiments that target **16** is first cleaved to some extent in the single-stranded region and only subsequently at the bulge. However, the absence of intermediate cleavage fragments and the fact that the overall rate of cleavage of **16** is greater than that of the perfectly matched target **17**, strongly indicates that the bulged phosphodiester is the favoured cleavage site. A plausible explanation is that the bulged nucleotides are constrained to conformations conducive to an intramolecular transesterification, i.e. an in-line displacement by a 2′-hydroxyl group (18). Alternatively, the bulge may simply

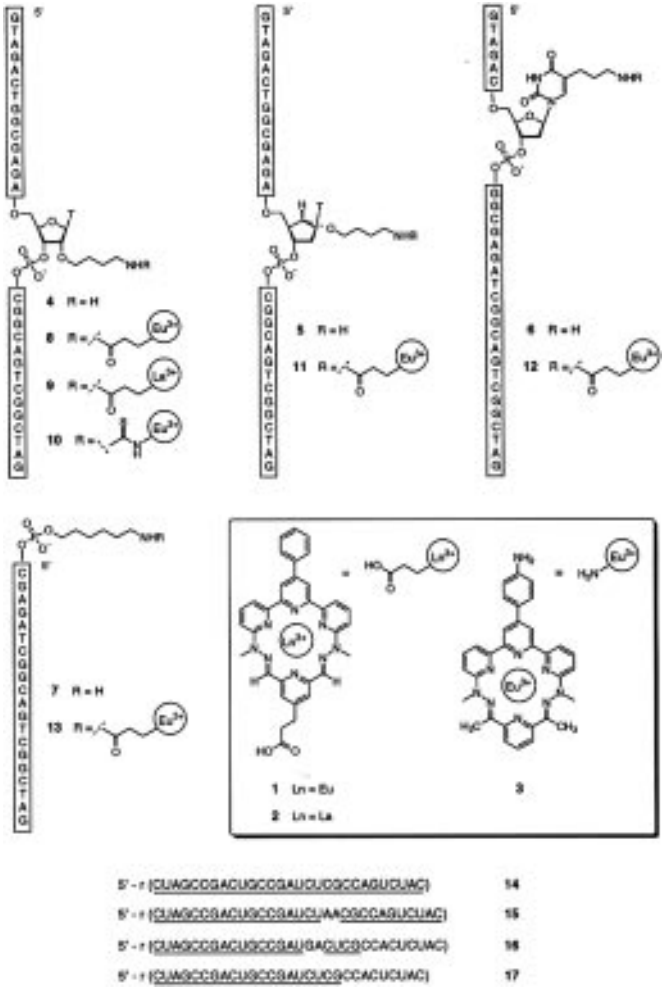

Figure 2. Lanthanide complexes, amino oligonucleotides, conjugates and ribooligonucleotide targets used in the present study. Bases which are complementary to the amino oligonucleotides **4–7** or conjugates **8–13** are underlined in oligoribonucleotides **14–17**; non-underlined bases give rise to single stranded overhangs or bulged ribonucleotides.

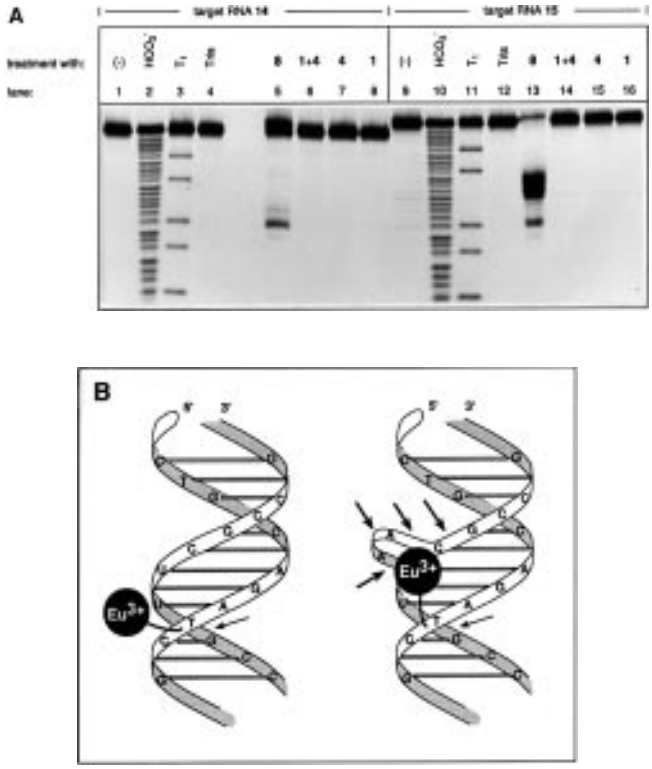

Figure 3. (**A**) Autoradiograph of a 12% denaturing polyacrylamide gel obtained after treatment of target oligoribonucleotides **14** and **15** (^{33}P labeled at the 5′-end) with conjugate **8**. Lanes 1 and 9, untreated RNA; lanes 2 and 10, alkaline hydrolysis (0.4 M HCO_3^-, pH 10, 70°C, 5 min); lanes 3 and 11, RNase T_1 partial digestion (0.2 U/µl, 70°C, 20 min); lanes 4 and 12, incubation in the reaction buffer (20 mM Tris–HCl, pH 7.4, 37°C, 16 h); lanes 5 and 13, treatment of **14** and **15**, respectively, with conjugate **8** (0.6 µM, pH 7.4, 37°C, 16 h); lanes 6–8 and 14–16, treatment of **14** and **15**, respectively, with metal complex **1** (0.6 µM) and/or amino oligonucleotide **4** (1 µM) under identical conditions (pH 7.4, 37°C, 16 h). (**B**) Schematic representation of cleavage sites (arrows) of oligoribonucleotides **14** (left) and **15** (right, with bulge) by conjugate **8**.

be a preferred binding site for the metal complex leading to a higher local concentration.

SUMMARY AND CONCLUSIONS

The experiments described above demonstrate the efficient sequence specific cleavage of RNA in the duplex region formed between the latter and a chemical ribonuclease. Cleavage is effected by the enforced presence of a bulge in the RNA target strand. The results can be summarised as follows: (i) RNA cleavage at double bulged nucleotides in the duplex region is seen with both endo- and exoconjugates; (ii) cleavage is not limited to a single site but occurs at several phosphodiesters within and adjacent to the bulge; (iii) most efficient cleavage is observed with endoconjugates reaching the bulge across the minor groove; (iv) less efficient cleavage was observed using an approach through the major groove, and (v) in a competition experiment, cleavage by an exoconjugate at the bulge was preferred over the single stranded region.

Since it is conceivable that cleavage within the duplex will result in sufficient destabilisation for product release, these findings represent a significant step in the development of true artificial ribonucleases capable of catalytic turnover.

MATERIALS AND METHODS

Preparation of oligonucleotides

Oligonucleotides were prepared by automated synthesis on a 1.5 µmol scale on a DNA synthesizer (Applied Biosystems Inc. 394A-08). DNA and 4-monomethoxy trityl (MMT) amino-C6 cyanoethyl phosphoramidites were obtained from MWG-Biotech GmbH, Expedite™ RNA cyanoethyl phosphoramidites from Millipore. Oligodeoxynucleotides **4–7** were cleaved from the support and base-deprotected by treatment with concentrated aqueous ammonia at 55°C overnight. After removal of the ammonia, the crude material was purified by HPLC using a semi-preparative RP-C18 column [Hypersil™, 5 µm particle size; 50 mM triethylammonium acetate (TEAA, pH 7.0) starting with 10% acetonitrile increasing with a gradient of 0.7%/min]. After concentration of the product-containing fractions, the trityl groups (DMT for **4–6**, MMT for **7**) were removed by treatment with 80% aqueous acetic acid for 30 min. The final purity of the amino oligonucleotides was assessed by CGE and PAGE. MALDI mass

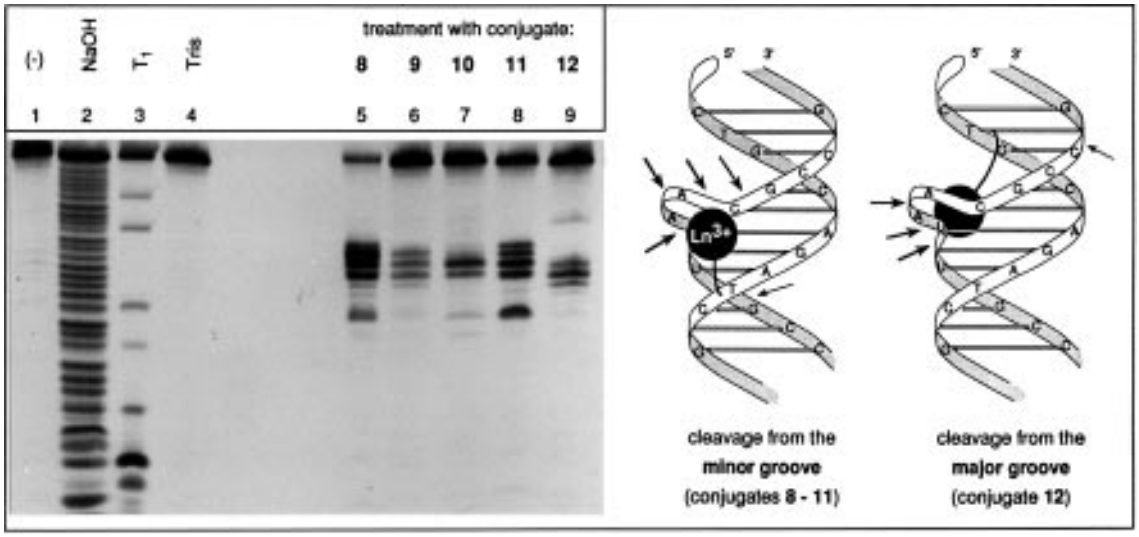

Figure 4. Autoradiograph of a 12% denaturing polyacrylamide gel obtained after treatment of target oligoribonucleotide **15** (^{33}P labeled at the 5′-end) with conjugates **8–12**. Lanes 1, 3 and 4, controls as described in Figure 3A; lane 2, alkaline hydrolysis (0.04 M NaOH, 70°C, 20 min); lanes 5–9, treatment of **15** with the conjugates (1 µM, pH 7.4, 37°C, 16 h) as indicated; sites of cleavage (arrows) by the different conjugates are illustrated on the right-hand side.

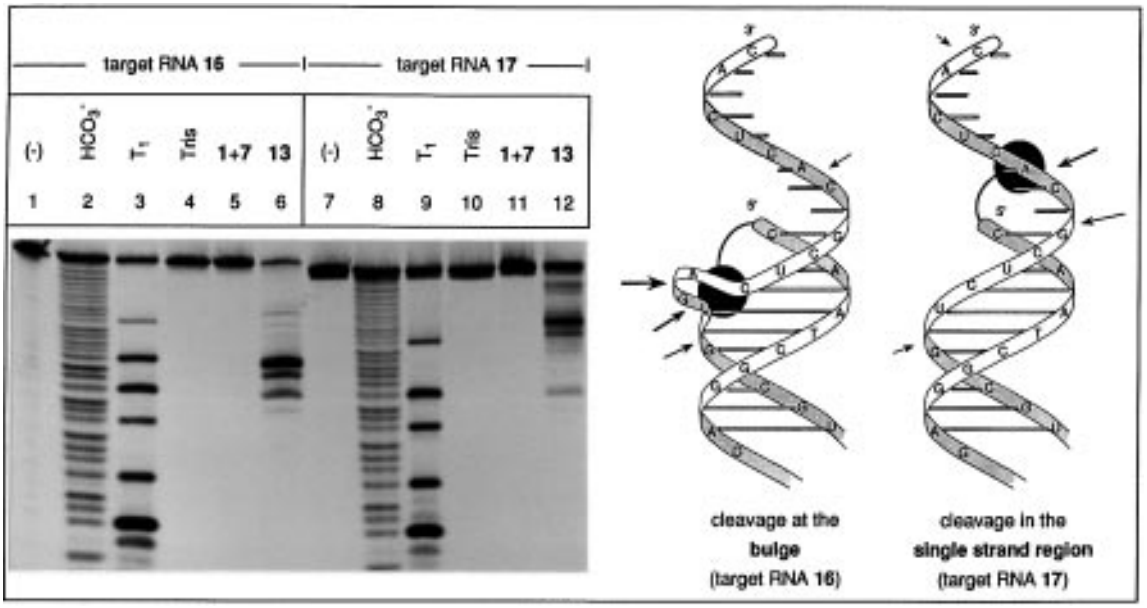

Figure 5. Autoradiograph of a 12% denaturing polyacrylamide gel obtained after treatment of target oligoribonucleotides **16** and **17** (^{33}P labeled at the 5′-end) with conjugate **13**. Lanes 1–4 and 7–10, controls as described in Figure 3A; lanes 5 and 11, treatment of **16** and **17**, respectively, with metal complex **1** (0.6 µM) and amino oligonucleotide **7** (0.6 µM) under identical conditions (pH 7.4, 37°C, 16 h); lanes 6 and 12, treatment of **16** and **17**, respectively with conjugate **13** (0.6 µM, pH 7.4, 37°C, 16 h); sites of cleavage (arrows) by the different conjugates are illustrated to the right.

spectra were obtained in either the positive or negative mode and are shown in Table 1.

Preparation of conjugates 8–13

Oligonucleotide conjugates were prepared according to the previously described procedure (4) using either the corresponding modified building blocks (14–16, conjugates **8–12**) or a 5′-terminal aminohexyl linker (conjugate **13**). Products were purified by RP-HPLC. The product purity was checked by CGE or PAGE. MALDI mass spectra were obtained and are shown in Table 1.

Oligoribonucleotides **14–18** were cleaved from the support and base-deprotected by treatment with a mixture of concentrated aqueous ammonia (25% v/v) in ethanol 16 h. The 2′-protecting group (*tert*-butyldimethylsilyl) was removed with a 1 M solution of tetrabutylammonium fluoride in tetrahydrofuran for 24 h at room temperature in the dark. The reaction mixture was quenched with an equal volume of 50 mM triethylammonium hydrogencarbonate (TAHC) solution (pH 7.0), dialysed against 7.5 mM TAHC (pH 7.0) at 4°C, concentrated and used for 5′-end labelling as described below.

Table 1. Mass spectral data of oligonucleotides

Cpd.	4[a]	5[a]	6[a]	7[b]	8[a]	9[b]	10[b]	11[a]	12[b]	13[b]
Calcd.	9112	9110	9065	6361	9812	9797	9841	9810	9767	7061
Found	9116	9115	9077	6363	9808	9800	9853	9813	9794	7065

[a]Mass spectrum measured in the positive ion mode.
[b]Mass spectrum measured in the negative ion mode.

RNA 5′-end labelling

Special precautions were made to prevent contamination by traces of metals and nucleases as described (19). Oligoribonucleotide **7** (100 pmol) was ^{33}P end-labelled by treatment with 5 U T4 polynucleotide kinase (Promega), in a solution (total volume 20µl) containing 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM spermidine, 50 mM Tris–HCl (pH 7.5) and 5 µCi [γ-^{33}P]ATP (Amersham, 1000 Ci/mmol). The mixture was incubated at 37°C for 30 min followed by ethanol precipitation. After addition of 15µl loading buffer (containing 0.025% xylene cyanol and 0.025% bromophenol blue in 80% deionised formamide and 7 M urea with 20 mM citric acid and 1 mM EDTA), the mixture was heated for 2 min at 95°C, chilled on ice and loaded on a 12% denaturing polyarylamide gel. After electrophoresis (2 h, 55 W) the RNA band was cut out, electroeluted and ethanol-precipitated. The material labelled by this procedure contained 200 000 c.p.m. (Cerenkov protocol).

RNA cleavage assay

5′-End labelled oligoribonucleotide **7** (12 000 c.p.m., final concentration estimated to 10–50 nM) and the corresponding oligonucleotide conjugate (600 nM final concentration) or complex (600 nM final concentration) were dissolved in 50 mM Tris–HCl buffer (pH 7.4; total reaction volume of 10 ml). The sample was heated for 1 min to 85°C, cooled to 37°C and kept at this temperature for 16 h. The reaction mixtures were diluted with 5 ml loading buffer, heated to 95°C for 1 min, chilled on ice and loaded on a 12% denaturing Long Ranger™ gel (AT Biochem). Electrophoretic separation (1.25 h, 60 W) was followed by exposure to X-ray film (Kodak, X-OMAT™ AR) and Phosphorimager™.

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