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Site-selective cleavage of structured RNA by a staphylococcal nuclease–DNA hybrid

(restriction endonuclease/hybrid enzyme/RNA cleavage)

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ABSTRACT **A hybrid enzyme consisting of an oligodeoxyribonucleotide fused to a unique site on staphylococcal nuclease site-selectively cleaves a number of natural RNAs including *Escherichia coli* M1 RNA (377 bases), 16S rRNA (1542 bases), and yeast $tRNA^{Phe}$. The oligonucleotide directs the nuclease activity of the enzyme to the nucleotides directly adjacent to the complementary target sequence on the substrate RNA. In the case of M1 RNA, hydrolysis occurs primarily at one phosphodiester bond, converting 50% of the starting material to product. Furthermore, the reaction products can be enzymatically manipulated: $tRNA^{Phe}$ was cleaved in the anticodon region and was religated to form the full-length tRNA in high yield. Because the specificity of these hybrid enzymes can be easily altered, they should prove to be useful tools for probing RNA structure and function.**

The availability of a wide number of restriction enzymes capable of hydrolyzing duplex DNA at defined sequences has tremendously increased our ability to manipulate DNA structure (1). In contrast, studies of RNA structure and function have been hampered by the failure to isolate an analogous class of RNA restriction enzymes from nature. To overcome this obstacle, strategies are being developed for site-selectively hydrolyzing RNAs at specific sites (2–6). One such strategy involves introducing a high degree of specificity into naturally occurring relatively nonspecific enzymes, which efficiently hydrolyze RNA. Oligodeoxyribonucleotide binding sites of defined sequence (7) have been linked near the active sites of the phosphodiesterases staphylococcal nuclease and RNase A to generate hybrid enzymes capable of selectively cleaving small (59–64 nucleotides; nt) relatively unstructured single-stranded DNA and RNA oligomers (8–10). We now report that hybrid enzymes can be constructed (Fig. 1) that are capable of site-selectively cleaving natural structured RNAs including *Escherichia coli* M1 RNA (the catalytic RNA component of RNase P), *E. coli* 16S ribosomal RNA, and yeast $tRNA^{Phe}$. The hybrid enzyme primarily hydrolyzes one phosphodiester bond of the 377-nt M1 RNA to yield two major products, which can be purified by electrophoresis. Moreover, because cleavage involves hydrolysis of the phosphodiester backbone, the cleavage products of the tRNA digestion can be ligated to re-form the intact tRNA molecule.

MATERIALS AND METHODS

Buffers were made with diethylpyrocarbonate-treated (0.1% vol/vol) water and were filtered through 0.22-μm sterile filters prior to use; pH values were determined at 25°C. Eppendorf tubes and other plasticware were autoclaved prior to use. Hybrid enzymes were synthesized and purified as

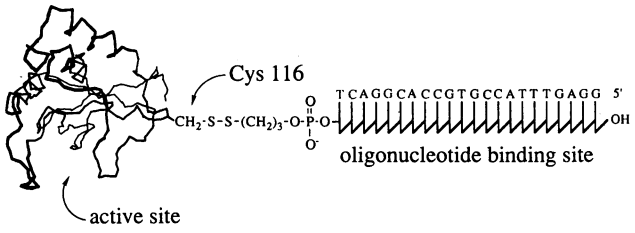

FIG. 1. Hybrid enzyme containing a mutant staphylococcal nuclease (Lys-116 to Cys-116) and a 3'-thiolated oligonucleotide.

described (8, 9). T4 polynucleotide kinase (11) and T7 RNA polymerase (12) were prepared as described; T4 RNA ligase was purchased from Takara Biomedicals, Japan. Melting temperature experiments (13) were performed with a Gilford 250 spectrophotometer in 50 mM sodium phosphate/50 mM NaCl/0.1 mM EGTA, pH 6.5; the temperature was increased from 10°C to 90°C at a rate of 1.0°C/min.

RNA Preparation. All RNAs were end-labeled on either the 5' termini with T4 polynucleotide kinase and [γ-^{32}P]ATP (14) or the 3' termini with T4 RNA ligase and 5'-[^{32}P]pCp (15). After purification from 7 M urea/5–8% polyacrylamide (acrylamide/bisacrylamide, 30:1) gels (16), the RNAs were recovered by elution overnight at 25°C in 0.5 M ammonium acetate/10 mM magnesium acetate/1 mM EDTA/0.1% NaDodSO$_4$, followed by extraction with an equal volume of chloroform/isoamyl alcohol (24:1) and two ethanol precipitations.

E. coli M1 RNA was obtained by runoff transcription of *Sna*BI-linearized plasmid pDW27 with T7 RNA polymerase (17). The RNA product was separated from unincorporated NTPs by anion-exchange chromatography on a Pharmacia Mono Q HR 5/5 column (9) prior to end-labeling. *E. coli* 16S rRNA was purified on a 1.5% low melting point agarose gel (16) from a mixture of 16S and 23S rRNA obtained from Boehringer Mannheim. The tRNA used in these studies was an amber suppressor yeast $tRNA^{Phe}_{CUA}$ prepared by the anticodon replacement method of Bruce and Uhlenbeck (18).

Site-Selective Cleavage Reactions. *M1 and 16S RNA.* Reaction mixtures in 8-μl volumes containing 1.6 pmol of ^{32}P-end-labeled RNA in 50 mM Bistris·HCl/50 mM NaCl/0.1 mM EDTA, pH 6.5, were incubated at 65°C for 1 min. The hybrid enzyme (1.0 pmol) in 1 μl of 5 mM Tris·HCl/1 mM EGTA, pH 7.5, buffer was then added, and the resulting solution was incubated at 65°C for 30 sec. After equilibrating the reaction mixture at the desired temperature for 1–2 min, the cleavage reaction was initiated by the addition of 1 μl of 20 mM $CaCl_2$ and quenched after 30 sec by the addition of 10 μl of 95% formamide/10 mM EGTA that had been preheated to 65°C. The quenched reaction mixture was immediately heated in a 90°C bath for 30 sec to fully denature the RNA followed by cooling on ice. The RNA fragments obtained

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Abbreviation: nt, nucleotide.

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were analyzed by electrophoresis on 7 M urea/6% polyacrylamide gels, followed by autoradiography using Kodak XAR 5 x-ray film. Band intensities were quantitated by excising the desired bands from the gel and counting them in (Beckman Ready-Safe) scintillation cocktail in an LKB (model 1209) liquid scintillation counter.

tRNA. Preparative reaction mixtures in 50-μl volumes containing 50 pmol (1 μg) of tRNA in 50 mM Tris·HCl/50 mM NaCl/0.1 mM EDTA, pH 7.0, were incubated at 65°C for 1 min. The hybrid enzyme (25 pmol) in 10 μl of 5 mM Tris·HCl/1 mM EGTA, pH 7.5, buffer was then added, and the resulting solution was incubated at 65°C for 1 min, followed by 15°C for 2 min. Cleavage was initiated at 15°C by the addition of 2.5 μl of 100 mM $CaCl_2$ and was quenched after 30 sec by the addition of 2.5 μl of 200 mM EGTA (pH 5.0), followed by immediate heating at 90°C for 30 sec. The reaction mixture was cooled to room temperature, and the RNA was concentrated by precipitation with ethanol. The two major reaction products were purified on a 12% denaturing polyacrylamide gel and were visualized by autoradiography or by staining with 0.02% toluidine blue. The product bands were excised from the gel, and the RNA was recovered as described above.

Religation of tRNA Fragments. The two tRNA half molecules obtained as described above were ligated as described (18). The 3′-end-labeled 3′ half of the tRNA (2.0 pmol) was incubated with a 5- to 10-fold excess of the unlabeled 5′ tRNA half molecule in 7 μl of water at 65°C for 2 min. The 3′-terminal phosphate groups were removed, and the 5′ terminus of the 3′ half of the tRNA was phosphorylated by treatment with T4 polynucleotide kinase at pH 7.0 (11): the solution was brought to a vol of 10 μl containing 20 mM Tris·HCl, 10 mM $MgCl_2$, 20 mM NaCl, 0.2 mM EGTA, 3.3 mM dithiothreitol, 30 μM ATP, and 10 μg of bovine serum albumin per ml (pH 7.0), after which 2.0 units of T4 polynucleotide kinase was added. After incubation at 37°C for 30 min, 1.0 unit of T4 RNA ligase was added and the reaction mixture was incubated an additional 60 min at 37°C. The ligation reaction was monitored by denaturing 8% polyacrylamide gel electrophoresis.

Hybrid Enzyme Stability. The 5′ hydroxyl group of the hybrid enzyme oligonucleotide binding site was phosphorylated with [γ-^{32}P]ATP and T4 polynucleotide kinase in the absence of dithiothreitol (due to the disulfide bond to the nuclease). To investigate the stability of the disulfide bond, the ^{32}P-end-labeled hybrid enzyme was incubated with 10 mM 2-mercaptoethanol in 50 mM Bistris·HCl/300 mM NaCl/0.1 mM EDTA, pH 7.0, buffer at 37°C, and the reduction of the disulfide was monitored by anion-exchange chromatography on a Pharmacia Mono Q HR 5/5 column (9).

RESULTS

Specificity. The specificity of hybrid enzymes constructed with 22-base oligonucleotide binding sites was compared to that of the free staphylococcal nuclease Lys-116 to Cys-116 mutant (K116C). Cleavage reactions were carried out with 0.16 μM ^{32}P-end-labeled M1 and 16S RNA substrates (pH 6.5; 65°C) using between 0.5 and 1.0 equivalent of hybrid enzyme. After hybridization of the RNA with the hybrid enzyme for 30 sec in the absence of Ca^{2+} ions, the cleavage reaction was initiated by the addition of Ca^{2+} at 65°C and was quenched after 30 sec by the chelation of the Ca^{2+} with EGTA.

Cleavage of M1 RNA by free staphylococcal nuclease (K116C) is relatively nonselective, giving rise to many products. In contrast, cleavage by the hybrid enzyme (binding site, 5′-GGAGTTTACCGTGCCACGGACT-3′) generates one major product with both 5′- and 3′-end-labeled RNA (Fig. 2 *Left*). The target site, nucleotides 217–238 of M1 RNA, consists of 15

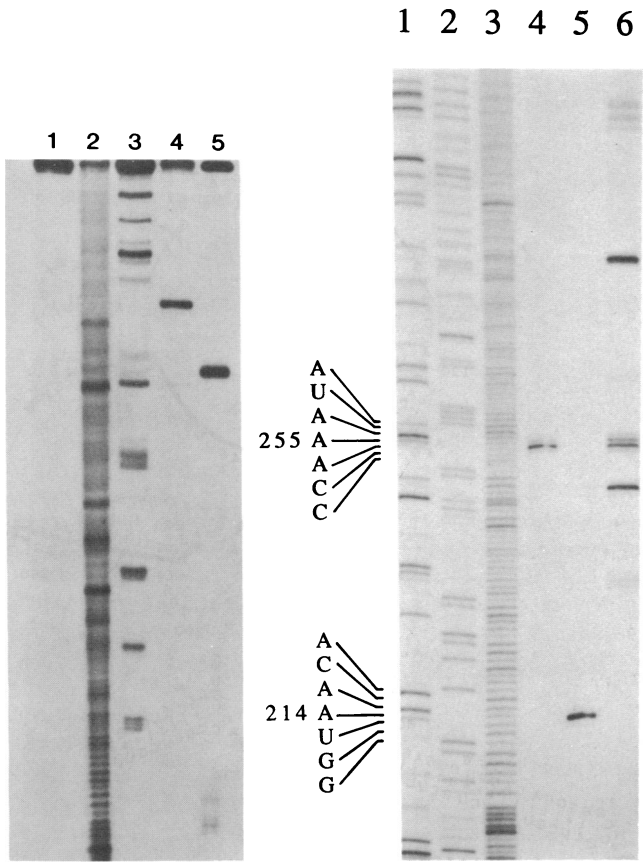

FIG. 2. (*Left*) Autoradiograph of a 6% denaturing polyacrylamide gel showing the site-selective cleavage of M1 RNA. Lanes: 1, 5′-end-labeled undigested RNA; 2, 5′-end-labeled hydroxide cleavage ladder; 3, 5′-end-labeled staphylococcal nuclease Cys-116 mutant digest; 4, 5′-end-labeled hybrid enzyme digest (target site A^{217}–C^{238}); 5, 3′-end-labeled hybrid enzyme digest (target site A^{217}–C^{238}). (*Right*) Autoradiograph of an 80-cm 7% denaturing polyacrylamide gel showing that hydrolysis occurs primarily at one phosphodiester bond. Lanes: 1, RNase U2 digest (A specific); 2, RNase T1 digest (G specific); 3, 5′-end-labeled hydroxide cleavage ladder; 4, 5′-end-labeled hybrid enzyme digest (target site A^{258}–C^{279}); 5, 5′-end-labeled hybrid enzyme digest (target site A^{217}–C^{238}); 6, 5′-end-labeled staphylococcal nuclease Cys-116 mutant digest.

nt of a putative single-stranded loop region and 7 nt of duplex RNA. An 80-cm 7% denaturing polyacrylamide gel revealed that hydrolysis occurs primarily at one phosphodiester bond, $A^{214}pA^{215}$ (Fig. 2 *Right*). The cleavage site is 2 bases away from the 5′-terminal A of the target sequence (Fig. 3) and located in a hairpin loop region of M1 RNA. A second hybrid enzyme (binding site, 5′-GGGCCGTACCTTATGAACCC-CT-3′) was constructed that binds nucleotides 258–279 of M1 RNA. This sequence corresponds largely to a putative double-stranded region of M1 RNA. Again, the hybrid enzyme cleaves primarily one phosphodiester bond, $A^{255}pA^{256}$, which is 2 bases away from the 5′-terminal A of the target sequence (Figs. 2 *Right* and 3).

Digestion of 5′-end-labeled *E. coli* 16S rRNA with the hybrid enzyme under the conditions described above (binding site, 5′-CTGCGTGCGCTTTACGCCCAGT-3′) yields a product (≈578 nt) consistent with cleavage occurring directly adjacent to the target site (Fig. 4). Although the location of the cleavage site could not be determined to nucleotide resolution, high sequence selectivity is observed in comparison to the cleavage pattern produced by the free nuclease in the presence or absence of the unattached 22-mer. As expected, the site-selective cleavage reaction is inhibited when the RNA is first hybridized to an excess of the

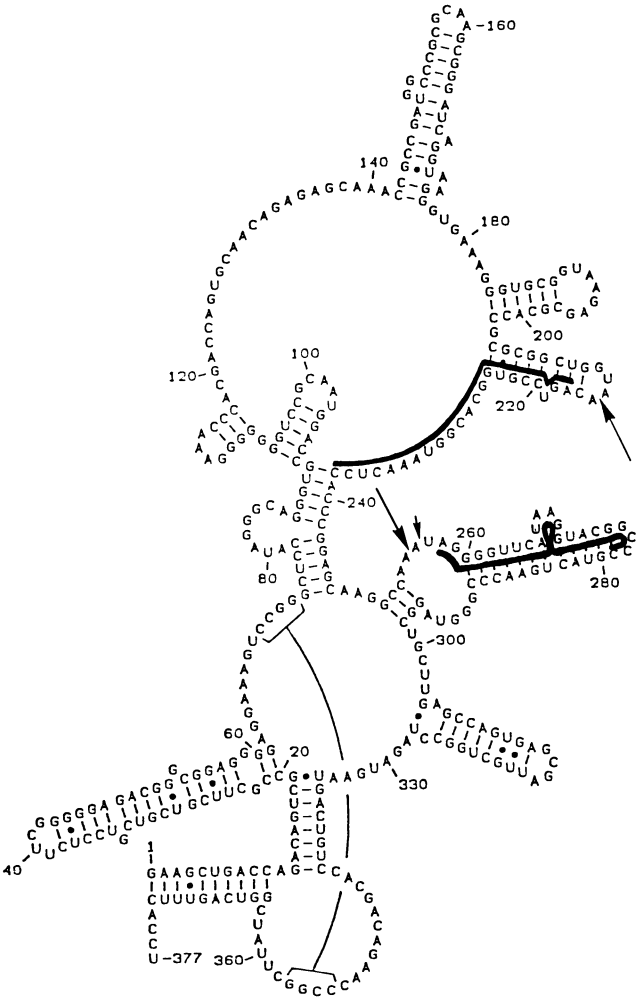

FIG. 3. Secondary structure map of *E. coli* M1 RNA, showing the results of two separate hybrid enzyme digestions. Solid lines denote the 22-base hybrid enzyme target sequences; arrows indicate the sites of cleavage: $A^{214}pA^{215}$ and $A^{255}pA^{256}$ (28).

free 22-mer. No selectivity is observed when either the M1 or 16S RNA and Ca^{2+} are mixed together prior to addition of the hybrid enzyme at 65°C, indicating that a hybridization step is essential. However, when a relatively unstructured 59-nt RNA is used as a substrate (9) and cleavage is initiated at 0°C, hybridization of a 14-nt hybrid enzyme prior to the addition of Ca^{2+} is not required for selective cleavage.

Reaction Conditions. Cleavage efficiency and specificity were examined under a variety of reaction conditions including various pH values, times, temperatures, and substrate/enzyme ratios. Reactions were carried out with 5'-end-labeled M1 RNA at a concentration of 0.16 μM and with a 22-nt hybrid enzyme. After separating the reaction products by denaturing polyacrylamide gel electrophoresis, three regions were cut out of the gel and analyzed in a scintillation counter: undigested starting material, site-selective cleavage product, and all other (nonselective) cleavage products. The optimal conditions are defined as those that yield the highest fraction of RNA starting material converted to the site-selective cleavage product.

The yield of the site-selective product is extremely pH dependent, with the optimum at pH 6.5 at 65°C and a cleavage time of 30 sec. At lower pH, there is little overall digestion, whereas, at higher pH (>7.5) nonselective cleavage becomes dominant. The reported pH optimum for staphylococcal nuclease is pH 9.5 (19), and we have shown that as the pH is lowered, the k_{cat} decreases and the K_m increases. The k_{cat} and K_m values for the hydrolysis of 16S plus 23S RNA by native

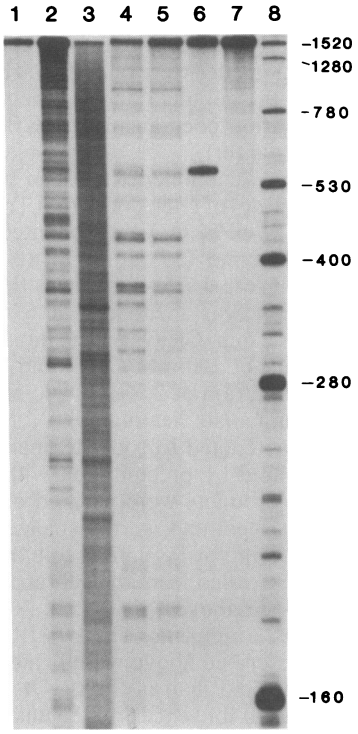

FIG. 4. Autoradiograph of a 6% denaturing polyacrylamide gel showing the site-selective cleavage of 5'-end-labeled 16S RNA. Lanes: 1, undigested RNA; 2, RNase U2 digest (A specific); 3, hydroxide cleavage ladder; 4, staphylococcal nuclease Cys-116 mutant digest; 5, Cys-116 mutant digest in the presence of an equimolar amount of the unattached 22-mer; 6, hybrid enzyme digest; 7, hybrid enzyme digest in the presence of excess unattached 22-mer; 8, RNA molecular weight markers (number of bases) (Bethesda Research Laboratories).

staphylococcal nuclease are 0.018 (ΔA_{260}/min)/(μg/ml) and 0.24 OD/ml, respectively, at pH 7.0, and 0.51 (ΔA_{260}/min)/(μg/ml) and 0.17 OD/ml, respectively, at pH 9.5 [as determined by the method of Cuatrecasas *et al.*(19)]. The optimum reaction time is 30 sec for cleavage at 65°C at pH 6.5. At

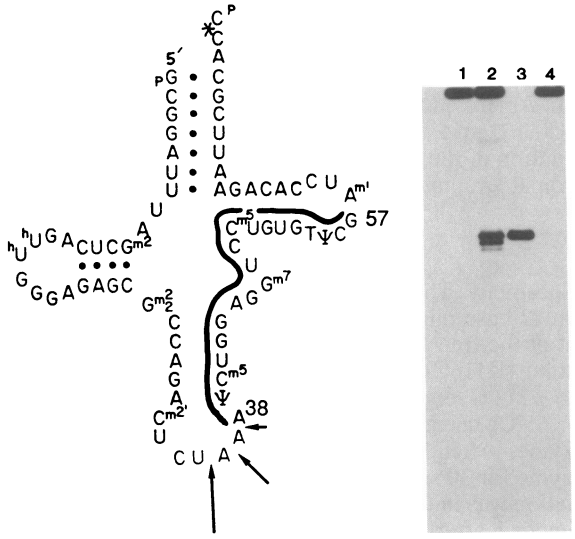

FIG. 5. (*Left*) Sequence of the tRNA; solid line denotes the 20-base target sequence; arrows indicate the sites of cleavage by the hybrid enzyme. (*Right*) Autoradiograph of an 8% denaturing polyacrylamide gel showing the site-selective cleavage and subsequent religation of 3'-end-labeled tRNA. Lanes: 1, undigested RNA; 2, hybrid enzyme digest; 3, gel-purified 3' half of tRNA; 4, ligation reaction mixture.

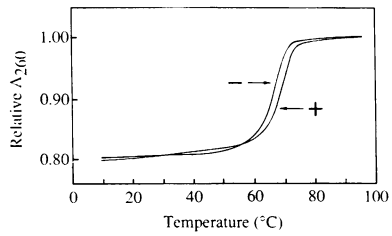

FIG. 6. Effect of nuclease on the stability of a DNA duplex. Absorbance melting curves of a 27-base oligodeoxyribonucleotide and a 22-base 3′-thiolated oligonucleotide binding site, with (+) and without (−) the attached nuclease.

shorter times, cleavage is site selective but product yields are low, and, at longer times, nonselective cleavage becomes significant. When the temperature of the digestion reaction is varied, at a hybridization time of 30 sec and a cleavage time of 30 sec (pH 6.5), an optimum is observed from 55 to 65°C. Cleavage specificity is distinctly lower at temperatures below 45°C. However, the optimum temperature for the cleavage reaction is dependent on the size of the oligonucleotide binding site, increasing with increasing length. The useful range of salt concentrations is between 50 and 300 mM NaCl, above which cleavage is specific, but yields of both selective and nonselective products are significantly lower. The maximum yield of selectively cleaved product occurred with reactions containing between 0.5 and 1.0 equivalent of hybrid enzyme per mol of RNA substrate, giving 50% site-selective product and 20% nonselective products.

Cleavage experiments with M1 RNA at 25°C with a hybrid enzyme containing an 11-mer oligonucleotide binding site (5′-TGCCACGGACT-3′) gave substantially lower yields of site-selective product than the corresponding 22-mer hybrid enzyme at the same concentration. Higher yields could be obtained only under conditions of excess hybrid enzyme, where nonselective cleavage became significant.

Religation. Since the cleavage mechanism of the nuclease involves the hydrolysis of the phosphodiester bond, the termini of the cleavage products can be enzymatically manipulated. This was demonstrated by the site-selective cleavage and subsequent religation of a tRNA. A yeast $tRNA^{Phe}$ was cleaved on a preparative scale with a hybrid enzyme containing a 20-nt binding site (5′-CGAACACAGGACCTC-CAGAT-3′) at 15°C after annealing at 65°C. The target sequence, nucleotides 38–57, corresponds largely to double-stranded regions of the tRNA. Cleavage occurs over a 3-nt region in the anticodon loop with most strand scission at $U^{35}pA^{36}$, which is 2 bases away from the 5′-terminal A of the target sequence (Fig. 5 *Left*). Both the 5′ and 3′ halves of the tRNA were recovered from a 12% denaturing polyacrylamide gel. After phosphorylating the 5′ ends and dephosphorylating the 3′ ends in one step by treatment with T4 polynucleotide kinase at pH 7.0 (11), the two RNA halves were ligated together with T4 RNA ligase (18) in >90% yield as determined by electrophoresis (Fig. 5 *Right*).

Hybrid Enzyme Properties. The hybrid enzyme was quite stable to heat, showing no loss of activity or specificity after incubation at 55°C for 15 min at pH 7.0 in the absence of Ca^{2+} ions. Stability of the hybrid enzyme toward reduction of the disulfide bond was also investigated: the end-labeled hybrid enzyme was incubated with 10 mM 2-mercaptoethanol (pH 7.0; 37°C) for various times. Reduction was complete after 45 min ($t_{1/2}$ = 5 min). Autolysis experiments with end-labeled hybrid enzyme and Ca^{2+} ion in the absence or presence of substrate revealed that <15% of the hybrid is degraded after 5 min.

The effect of the nuclease on the ability of the oligonucleotide to form a stable duplex was investigated by measuring the absorbance melting curves of the free oligonucleotide binding site and of the hybrid enzyme with a complementary DNA 27-mer (5′-GCGCGAGTCCGTGGCACGGTAAACT-CC-3′) in the absence of calcium ions. Extra bases were included at the 5′ termini of the target sequence so as to detect any interaction between the nuclease and the complementary strand. The melting temperatures of a free 11-mer (50°C) and free 22-mer (67°C) complexed to the 27-mer were essentially the same as those of the corresponding hybrid enzymes (Fig. 6).

DISCUSSION

Hybrid enzymes consisting of an oligonucleotide fused to Cys-116 of a staphylococcal nuclease mutant (K116C) have been shown to site-selectively hydrolyze a number of natural RNAs. M1 RNA was cleaved primarily at one position, $A^{214}pA^{215}$, in a hairpin loop by a 22-nt hybrid enzyme that binds largely to a putative single-stranded region of M1 RNA. A second hybrid enzyme (22 nt) that binds a putative duplex region of M1 RNA also cleaved the RNA primarily at one position, $A^{255}pA^{256}$. *E. coli* 16S RNA was cleaved with high selectivity using a 22-nt hybrid enzyme, although the cleavage site could not be determined to nucleotide resolution because of the large size of the cleavage fragments. Cleavage of a tRNA in the anticodon loop using a 20-nt hybrid enzyme that binds primarily to stem regions of the tRNA resulted in hydrolysis of three phosphodiester bonds with most strand cleavage at $U^{35}pA^{36}$. Specific cleavage of RNA by the hybrid enzymes appears to be effective with a variety of secondary structures; the primary requirement for cleavage is an A+U-rich cleavage site.

It was shown previously that relatively short (59–62 nt) unstructured synthetic RNA oligomers could be cleaved by hybrid enzymes with 14-nt binding sites and without prior hybridization (9, 10). However, attempts to cleave larger natural RNAs without prehybridization resulted in low yields of site-selective product. Therefore, cleavage reactions were carried out by prior hybridization of the hybrid enzyme to the target RNA at 65°C (in the absence of Ca^{2+}), where some of the RNA secondary and tertiary structure can be melted out (20–22). Since staphylococcal nuclease is rather stable to thermal denaturation (19, 23), an oligonucleotide binding site with a melting temperature (t_m) of ≈65°C was used to deliver the nuclease to the target sequence. Hybridization at temperatures lower than 37°C did not result in site-selective cleavage. Cleavage of M1 RNA with an 11-nt hybrid enzyme gave site-selective cleavage, but in lower yields with more nonselective hydrolysis relative to the corresponding 22-nt hybrid enzyme. These results suggest that the yield of selective cleavage product depends on the relative stability of the DNA·RNA hybrid compared to the stability of the local substrate RNA structure. The high selectivity of the hybrid enzyme with these natural RNAs most likely results from a combination of the high specificity of DNA·RNA hybridization and the inherent specificity of the nuclease [which prefers A+U-rich regions (19)].

The position of the primary cleavage site relative to the hybridization site of the enzyme typically occurs 2 or 3 bases away from the 5′-terminal A of the target sequence. The oligonucleotide binding domain is tethered to the enzyme by a flexible linker, ensuring that the inherent specificity of staphylococcal nuclease is accommodated and that the tethered binding and hydrolytic domains remain functional. The independence of the binding properties of the oligonucleotide from the presence of the nuclease is suggested by the fact that the t_m of a 22-nt hybrid enzyme with a complementary target DNA is virtually identical to that of the free oligonucleotide (Fig. 6). In some cases, especially RNAs lacking substantial secondary and tertiary structure, the hybrid enzyme can cleave 4 or 5 phosphodiester bonds at the target site with

similar efficiencies (9), suggesting that there is considerable conformational freedom in such RNA, allowing accessibility of the nuclease to many sites.

Cleavage of RNA by the hybrid is relatively sensitive to reaction conditions. Highest specificity is obtained at lower pH values, corresponding to decreased values of k_{cat}/K_m for native staphylococcal nuclease. We have also shown (unpublished results) that hybrid enzymes constructed from mutants with decreased k_{cat}/K_m values cleave single-stranded DNA substrates with higher specificity than the K116C hybrid enzyme (D. R. Corey, personal communication). Nonselective cleavage by the hybrid enzyme can result either from cleavage by nonhybridized enzyme, cleavage by autolyzed hybrid enzyme, or secondary cleavage by hybrid enzyme still associated with the hydrolyzed target sequence. The yield and composition of the nonselective cleavage products obtained with the M1 specific hybrid enzyme are similar to those obtained with a noncognate (16S) hybrid enzyme under the same conditions. These results are consistent with the notion that nonselective cleavage results from enzyme–RNA complexation that is independent of the tethered oligonucleotide. One might therefore expect that a decreased k_{cat}/K_m will reduce the rate of nonselective (hybridization independent) cleavage relative to the rate of selective cleavage, where the enzyme is preassociated with the target RNA via the oligonucleotide binding site. Cleavage specificity and efficiency are highest at shorter (30 sec) reaction times.

Under optimal conditions, M1 RNA was digested with one equivalent of hybrid enzyme to yield 50% site-selective product and 20% nonselective products. Product yields could be improved by annealing the hybrid enzyme to RNA, carrying out the cleavage reaction, quenching with EGTA, reannealing, and carrying out the reaction again. Attempts at finding conditions where the K116C hybrid enzyme would carry out multiple turnovers with high selectivity were unsuccessful. Nonetheless, due to the ease of synthesis of the hybrid enzymes, they can be readily used as stoichiometric reagents for most RNA cleavage experiments. It may be possible, however, to construct hybrid enzymes capable of multiple turnover by the use of nonhydrolyzable oligonucleotide binding sites, by the use of an RNase A hybrid enzyme (which cannot autolyze), or by the use of k_{cat}/K_m staphylococcal nuclease mutants [this has in fact been shown to be the case when DNA is cleaved by a Tyr-113 to Ala-113 (Y113A), K116C 15-nt hybrid enzyme (D. R. Corey, personal communication)].

The utility of hybrid enzymes for the sequence-specific manipulation of RNA was demonstrated by the site-selective cleavage of a tRNA in the anticodon loop, followed by gel purification and ligation of the two half molecules to regenerate the full-length tRNA. Hydrolysis by the nuclease leaves 3′ phosphate and 5′ hydroxyl termini as opposed to a number of other nucleic acid cleavage strategies (24–27). Consequently, addition of T4 polynucleotide kinase and T4 RNA ligase leads to efficient religation of RNA cleaved by the hybrid enzyme.

In conclusion, hybrid enzymes have been constructed that are capable of site-selectively cleaving natural RNAs to produce fragments that can be isolated and enzymatically manipulated. Because the hybrid enzymes can be rapidly synthesized with a wide variety of binding specificities, they should prove useful as tools for studying RNA structure and function. It also should be possible to develop strategies for delivering these enzymes to large duplex DNAs, perhaps by using recA or by replacing the oligomer binding site with DNA binding proteins.

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