Sequence-specific cleavage of RNA by Type II restriction enzymes

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ABSTRACT

The ability of 223 Type II restriction endonucleases to hydrolyze RNA–DNA heteroduplex oligonucleotide substrates was assessed. Despite the significant topological and sequence asymmetry introduced when one strand of a DNA duplex is substituted by RNA we find that six restriction enzymes (AvaII, AvrII, BanI, HaeIII, HinfI and TaqI), exclusively of the Type IIP class that recognize palindromic or interrupted-palindromic DNA sequences, catalyze robust and specific cleavage of both RNA and DNA strands of such a substrate. Time-course analyses indicate that some endonucleases hydrolyze phosphodiester bonds in both strands simultaneously whereas others appear to catalyze sequential reactions in which either the DNA or RNA product accumulates more rapidly. Such strand-specific variation in cleavage susceptibility is both significant (up to orders of magnitude difference) and somewhat sequence dependent, notably in relation to the presence or absence of uracil residues in the RNA strand. Hybridization to DNA oligonucleotides that contain endonuclease recognition sites can be used to achieve targeted hydrolysis of extended RNA substrates produced by in vitro transcription. The ability to ‘restrict’ an RNA–DNA hybrid, albeit with a limited number of restriction endonucleases, provides a method whereby individual RNA molecules can be targeted for site-specific cleavage in vitro.

INTRODUCTION

The discovery of Type II restriction endonucleases (REases) (1) with their capacity to introduce sequence-specific double-strand breaks in complex DNA molecules (2) and the use of DNA ligase to reconnect such molecules in predictable and user-defined combinations (3) provided the key enabling technologies of in vitro DNA manipulation and the subsequent biotech revolution. As of June 2010, gene sequences encoding Type II REases that recognize 313 different target DNA sequences have been determined and are listed in the restriction enzyme database (REBASE) (4). Genes encoding different Type II REases tend to be highly divergent at the level of primary sequence and, with the exception of isoschizomers, enzymes that recognize the same sequence and cleave DNA at identical positions, are not reliably identifiable using standard bioinformatic approaches (5). As a consequence, restriction enzymes are classified according to combinations of functional properties rather than on the basis of genetic relatedness (6). X-ray crystallographic analyses, however, reveal underlying structural relationships that define, on the basis of conserved active site architectures, five distinct groups of Type II REases (5,7,8). Members of three classes have an absolute requirement for divalent cations in order to catalyze DNA cleavage while the remaining two appear to hydrolyze their substrates using metal-independent mechanisms (5). More recently, the available repertoire of REases has further expanded to include modified restriction enzymes with improved cleavage fidelity (Z. Zhu et al., 2009. United States Patent Application 20090029376) or with redesigned cleavage-site specificity (9,10) created through the successful application of protein engineering techniques.

With the exception of analyses of the inhibitory effects of base methylation, by the cognate S-adenosylmethionine-dependent DNA methyltransferases (MTases) that protect the host genomes of REase-expressing prokaryotes in vivo, few published studies have addressed the capacity of restriction enzymes to cleave chemically modified DNA substrates. Those that have are commonly directed towards answering specific questions about particular enzymes, for example, the use of phosphorothioate-substituted substrates to determine the stereochemistry of phosphodiester bond hydrolysis (11,12) or assessing the different effects of various modified bases and backbone architectures on the hydrolytic reactions catalyzed by a pair of neoschizomeric enzymes (13). Notable exceptions include testing the activity of fourteen different Type II
enzymes on substrates that include mismatched bases within their recognition sites (14) and a systematic in vitro analysis of the effects of adenine and cytosine methylation on the activities of a large number of REases [Stickel, S. K. and Roberts, R. J., unpublished results available in REBASE (4)]. Such analyses show that some restriction enzymes are able to cleave atypical DNA substrates in vitro, including some containing chemical modifications that they would not be expected to encounter in vivo. With this in mind, we considered the possibility that some Type II REases might catalyze the sequence-specific hydrolysis of the other extant biological polynucleotide, RNA.

The only published experimental analysis of REase activity on RNA–DNA substrates is a very early study by Molloy and Symons using cDNA–RNA heteroduplexes produced from SAT RNA and RNA 4 of cucumber mosaic virus (15). Eight enzymes were identified that appeared to cleave the DNA strand and, in the case of HaeIII and TaqI, indirect evidence of possible RNA strand hydrolysis was presented. Given the available methods at that time, however, it was not possible to confirm sequence-specific cleavage of the RNA strand or to exclude contaminating ribonuclease activity as a source of any observed activity (Molloy, P. M. and Roberts, R. J., unpublished data). Subsequently it was shown that a single ribonucleotide positioned 3’ of the scissile bond in a DNA duplex blocked cleavage by EcoRI and BamHI, whereas the latter enzyme was partially active on a duplex containing a ribonucleotide on the 5’-side of the target phosphodiester (16,17). During almost 30 years since the Molloy and Symons paper was published, there have—to the best of our knowledge—been no further attempts to identify REases with ribonuclease activity.

From a structural perspective RNA might appear to be an unlikely surrogate substrate for restriction enzymes, as the presence of a 2'-hydroxyl group adjacent to the scissile phosphodiester linkage can present a steric barrier to assembly of a catalytically competent complex with an enzyme that has evolved to bind and hydrolyze a substrate that lacks such a group (16). Also, crystallographic analyses of RNA–RNA homoduplexes and RNA–DNA heteroduplexes show that the presence of the 2'-hydroxyl group causes both to adopt an A-form helical structure characterized by an expanded minor groove and contracted major groove relative to canonical B-form DNA (18,19). As sequence-specific recognition by Type II REases commonly involves precise contacts between amino acid side chains and the edges of nucleotide bases in the major groove, such interactions are likely to be unavailable, at least in part, to an equivalent RNA substrate (20) However, structural studies of RNA–DNA heteroduplexes in solution suggest that the DNA strand conformation is intermediate between A and B-forms (21) and crystal structures of Bacillus halodurans RNase H and human RNase H1 bound to such a heteroduplex show the RNA and DNA strands adopt A-like and B-like conformations, respectively (22,23). Furthermore, A-form conformational motifs are observed in co-crystal structures of duplex DNA bound to many DNA-binding proteins (including homing endonucleases and REases; 24) although others suggest that some such assignments are predicated on an unduly restrictive definition of B-form DNA (25).

Here we report results obtained from activity screening of a large number of different Type II REases using fluorophore-tagged RNA–DNA duplex oligonucleotide substrates. We identify a small subset of restriction enzymes that catalyze hydrolysis of such substrates and which may prove useful as sequence-specific RNA cleavage reagents.

MATERIALS AND METHODS

All REases and endonuclease reaction buffers, BSA, Phusion HF DNA polymerase, calf intestinal phosphatase (CIP), T4 polynucleotide kinase and T4 RNA ligase 2, were obtained from New England Biolabs (Ipswich, MA, USA). It should be noted that the TaqI used in these experiments is the recombinant enzyme, rather than the native enzyme used by Molloy and Symons, and differs slightly in amino acid sequence. Synthetic oligonucleotides and oligoribonucleotides were produced by the New England Biolabs Organic Synthesis Division or purchased from Integrated DNA Technologies (Coralville, IA, USA). Reagents for in vitro transcription (IVT) using T7 RNA polymerase, RNase-free distilled and deionized H2O and RNase-free TE buffer were purchased from Ambion/Applied Biosystems (Austin, TX, USA). Reagents for polyacrylamide gel electrophoresis of nucleic acids were obtained from Invitrogen (Carlsbad, CA, USA).

Initial screening experiments

Unlabeled and 3’-FAM- or 5’-FAM-tagged synthetic oligonucleotides or oligoribonucleotides were dissolved in RNase-free water or TE. Concentrations, normalized to the theoretical extinction coefficient at 260 nm for each sequence, were determined spectrophotometrically. Table 1 lists the sequences of oligonucleotides and oligoribonucleotides used to generate the data presented in this article and a complete list of oligonucleotides and oligoribonucleotides used in initial screening experiments is also provided in Supplementary Table S1. Annealing reactions (50 μl), including 50 pmol of each complementary strand in NEBuffer 4 (20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol), were heated at 90°C for 5 min and cooled slowly to room temperature. Endonuclease assays (20 μl) included 1–5 pmol of substrate duplex, with or without 1–2 μl of REase, and were incubated for 3 or 4 h using the NEBuffer (supplemented with 0.1 mg/ml BSA where appropriate) and temperature recommended for each enzyme. Reaction products were resolved by electrophoresis in 20% polyacrylamide/TBE native or 15% polyacrylamide/urea/TBE denaturing pre-cast gels (Novex) and visualized by UV transillumination.

Strand-specific analysis of cleavage products

Enzymes that showed evidence of heteroduplex cleavage in the initial screening were reanalyzed using substrates in...
which the RNA and DNA strands were labeled at their 5'-ends with FAM or TAMRA fluorophores, respectively. Oligonucleotides and oligoribonucleotides were dissolved in RNase-free water at a concentration of 100 μM. Annealing reactions (0.1 ml) routinely included 10 μM of each RNA or DNA strand yielding a nominal duplex concentration of 10 μM. All restriction assays (0.1 ml) used NEBuffer 4 (supplemented with 0.1 mg/ml BSA) and contained 10 nM or 20 nM (1 or 2 pmol) duplex substrate. Reaction products were mixed with four volumes of denaturing gel-loading buffer (95% w/v formamide, 25 mM EDTA, 0.05% w/v Orange-G dye), heated to >95°C for 2 min and aliquots (20 or 40 fmol) were resolved by electrophoresis in 15% polyacrylamide/urea/TBE denaturing pre-cast gels (Novex).  

FAM (RNA) and TAMRA (DNA) fluorescence was monitored by gel scanning using a Typhoon 9400 variable mode imager (GE Healthcare, Piscataway, NJ, USA). Excitation wavelengths of 488 nm (with 520 nm BP40 filter) and 532 nm (with 580 nm BP30 filter) were used for FAM and TAMRA scans, respectively. Data were collected at 25, 50 or 100 μm resolution with a single scan for each fluorophore using PMT settings of 300–500 V. Scan data were analyzed with ImageQuant software (GE Healthcare, Piscataway, NJ, USA). Both the FAM and TAMRA scans for each gel were displayed using the high-contrast option in ImageQuant. When creating dual-color (superimposed) images the brightness of individual scans was adjusted with ImageQuant to compensate for the differing fluorescence yields of the two fluorophores. Images (.bmp files) were exported from ImageQuant to Adobe Photoshop for RGB to CMYK conversion (and cropping) prior to inclusion in the figures.

### Specificity of RNA and DNA strand cleavage

Restriction products of DNA–DNA and equivalent RNA–DNA duplexes were analyzed by denaturing-PAGE as described. 5'-FAM labeled RNA oligonucleotides corresponding to the predicted products of cognate RNA cleavage were used as controls. The sizes of endonuclease cleavage products and control RNAs of cognate RNA cleavage were used as controls. The sizes of endonuclease cleavage products and control RNAs of cognate RNA cleavage were used as controls. The sizes of endonuclease cleavage products and control RNAs of cognate RNA cleavage were used as controls. The sizes of endonuclease cleavage products and control RNAs of cognate RNA cleavage were used as controls.

### Relative activity of REases on different substrates

REase specific activities were determined using highly concentrated, BSA-free, enzyme stocks. Enzyme monomer concentration was determined.
spectrophotometrically based on the calculated extinction coefficient at 280 nm for each REase. The 2-fold dilution series of each enzyme were assayed using three different substrates: bacteriophage λ DNA (1 μg); TAMRA-labeled DNA–DNA homoduplex (1 pmol); and TAMRA/FAM-labeled RNA–DNA heteroduplex (1 pmol). All reactions used NEBuffer 4 (supplemented with BSA) and were incubated for 1 and 3 h for DNA–DNA and RNA–DNA substrates, respectively. λ-DNA restriction products were resolved by agarose gel electrophoresis whereas fluorescent oligonucleotide and oligoribonucleotide cleavage products were analyzed by denaturing-PAGE and fluorescence scanning as described earlier.

**Oligonucleotide-directed cleavage of in vitro-transcribed RNA**

Two 116-mer oligonucleotides containing 32-nt self-complementary 3′-termini (IM433D and IM434D, Table 1) were mixed together and extended by Phusion HF polymerase in the presence of 0.2 mM dNTPs to produce a 200-bp DNA product. An aliquot of this DNA was then reamplified using 25 cycles of PCR using the same polymerase, and 26-mer flanking oligonucleotides, to produce a template for IVT which contains a T7 promoter sequence followed by 175 bp of DNA that includes unique AvaII, AvrII, BanI, Hinfl and TaqI restriction sites. IVT employed the T7 Megascript Kit (Ambion, Austin, TX, USA), using conditions recommended for the synthesis of short transcripts. Reaction products were treated with Turbo DNAse (Ambion) to degrade the template DNA, followed by extraction with phenol:chloroform:isoamyl alcohol (PCI, 25:24:1) and precipitation with ammonium acetate and isopropanol. Precipitated nucleic acids, comprising RNA ligation products (plus excess oligonucleotide and oligoribonucleotide) were pelleted by centrifugation, washed with 70% (w/v) ethanol, dried using a Speed-Vac centrifuge and redissolved in 0.1 ml RNase-free water. An aliquot of the original IVT template PCR product was reamplified with Phusion HF polymerase using a FAM-labeled oligonucleotide to produce a 5′-FAM-tagged DNA duplex with an identical sequence to the RNA product of the splinted ligation reaction.

Samples of the FAM-labeled RNA ligation product (3.2 pmol) were mixed with a 2-fold molar excess of one of five different DNA oligonucleotides designed to hybridize with the RNA at the unique AvaII, AvrII, BanI, Hinfl or TaqI restriction sites. Annealing reactions (20 μl in RNase-free water) were carried out by heating the RNA/oligonucleotide combinations to 90°C for 2 min, followed by rapid cooling to 4°C using a thermocycler with a heated lid to minimize evaporation. Restriction analyses used 5 μl of heteroduplex product (0.8 pmol RNA) in 10 μl reactions containing NEBuffer 4 plus 1 μl of the cognate REase and were incubated at 37°C for 1 h. Ligation reaction products were mixed with 40 μl of denaturing gel-loading buffer and resolved by denaturing PAGE as described for the oligonucleotide heteroduplex cleavage experiments. As previously, FAM (RNA) fluorescence was monitored by gel scanning using the Typhoon 9400 variable mode imager.

**RESULTS**

**Initial screening experiments**

A total of 223 REases (and four nicking endonucleases) were screened for the ability to cleave RNA–DNA heteroduplex and control substrates as described above. A complete list of the enzymes tested during this initial screening process is presented in Supplementary Table S2. Assays involved extended incubation (3–4 h) using 1–2 μl of enzyme which corresponds to 4–100 U depending upon the stock concentration of the individual REase. These analyses identified four REases that appeared to cleave both DNA and RNA strands of a heteroduplex substrate to some extent (Figure 1). These enzymes are AvaII (cut site G/GWCC where W = A or T), AvrII (cut site C/CTAGG), BanI (cut site G/GYRCC where Y = C or T and R = A or G) and TaqI (cut site T/CGA). Assays using REase Hinfl (cut site G/ANTC where N = A, C, G or T) efficiently cleaves only the DNA strand of an RNA–DNA heteroduplex (Figure 1).
AvaII, AvrII, BanI and TaqI endonucleases cleave both strands of an RNA–DNA heteroduplex substrate

Oligonucleotide IM398D and the reverse-complementary oligoribonucleotide IM399R were designed to include restriction sites for each of the five REases identified in the initial screening experiments that showed evidence of RNA-strand hydrolysis (Table 1). As IM398D and IM399R contain 5'-TAMRA and 5'-FAM fluorophores, respectively, a heteroduplex substrate formed from these two oligomers permits simultaneous monitoring of REase cleavage of both strands. Figure 2B shows the results of denaturing-PAGE analysis of the products of assays containing 1 pmol of IM399R–IM398D substrate and two different quantities of AvaII, AvrII, BanI and TaqI endonucleases. Each REase evidently cleaves both substrate strands to yield defined RNA and DNA fragments. If such cleavage occurs at the canonical positions within the target sequence then the predicted sizes of cleavage products for the DNA strand are 20, 24, 27 and 31 nucleotides for TaqI, AvaII, AvrII and BanI, respectively. Conversely, those for the RNA strand are 13, 17, 21 and 26 ribonucleotides for BanI, AvrII, AvaII and TaqI, respectively. Inspection of Figure 2B suggests that the observed electrophoretic mobilities of the different DNA and RNA fragments appear, to a first approximation, to be consistent with the predicted products of cognate cleavage.

Increasing the DNA:RNA ratio in substrate annealing drives RNA-strand cleavage towards completion

For REase-catalysed hydrolysis of RNA–DNA heteroduplexes to be of any practical utility requires that the RNA strand cleavage be both specific and efficient. REase TaqI cleaves both strands of an RNA–DNA heteroduplex substrate to completion (Figure 2B, lanes 8 and 9). However, the reactions catalyzed by AvaII, AvrII and BanI presented in Figure 2B did not result in complete cleavage of the substrate duplex, a significant proportion...
of the RNA strand remaining intact in each case. This might reflect different intrinsic cleavage rates for the RNA and DNA strands, the presence of insufficient REase in the assay, an excess of RNA strand in the annealing reaction or a subpopulation of annealed products that are refractory to cleavage by these enzymes. We reasoned that the first two potential problems might be addressed by the simple expedient of adding more REase to the reaction and the latter two by using an excess of DNA strand in the annealing reaction. To this end, IM399R and IM398D were annealed at molar ratios (RNA:DNA) of 1:1 or 0.5:1—the former being equivalent to the substrate utilized in the earlier assays. Aliquots of each containing 1 pmol DNA strand were incubated with REases as previously, except that the number of enzyme units was increased 2- (AvaII), 3- (AvrII) or 10-fold (BanI) compared to the highest concentrations used in the prior experiments. Products of these assays were resolved by denaturing-PAGE then visualized using the Typhoon Imager and the results are presented in Figure 2B.

Increasing the enzyme concentration without changing the RNA:DNA ratio has a significant beneficial effect in the case of BanI (compare lane 4 of Figure 2C with lane 6 of Figure 2B) but yields only modest improvement in the capacity of AvaII (lane 2 in Figures 2C and 2B) and AvrII (Figure 2C, lane 3 and Figure 2B, lane 4) to cleave the RNA strand. The combination of increased enzyme concentration and reduced RNA:DNA ratio, however, results in near-complete cleavage of the RNA strand by all three enzymes (compare lanes 6, 7 and 8 with lanes 2, 3 and 4 in Figure 2C).

Specificity of phosphodiester bond cleavage in RNA–DNA heteroduplex substrates

Oligonucleotide IM397D (Table 1) is a 5'-TAMRA-labeled DNA equivalent of oligoribonucleotide IM399R. Thus, REase cleavage of an IM397D-IM398D homoduplex or IM399R–IM398D heteroduplex will yield identical IM398D-derived products if the same phosphodiester bonds are hydrolyzed in both substrates. As no equivalent controls are available for the IM399R-derived RNA products of heteroduplex cleavage, 5'-FAM-labeled oligoribonucleotides IM401R to IM404R (Table 1) were synthesized based on the predicted cleavage products of IM399R using REases BanI, AvrII, AvaII and TaqI, respectively. The DNA–DNA and RNA–DNA substrates were each incubated with AvaII, AvrII, BanI and TaqI REases and the cleavage products resolved by denaturing-PAGE alongside the relevant synthetic oligoribonucleotide product controls. The scanned image of this gel is presented in Figure 3A and reveals an almost exact concordance between the electrophoretic mobilities of the equivalent DNA products derived from each substrate and enzyme combination. Similarly the RNA products of RNA–DNA heteroduplex cleavage by each enzyme align precisely with the relevant synthetic RNA controls. Aliquots of all samples shown in Figure 3A were subsequently resolved at single nucleotide resolution by capillary electrophoresis. The fragment analysis data for the AvaII restriction products and synthetic RNA control are shown in Figure 3B and confirm that the REase cleaves both strands of an RNA–DNA heteroduplex substrate by AvaII, AvrII, BanI and TaqI restriction enzymes. (A) Sequence of IM399R–IM398D heteroduplex substrate with boxes defining REase recognition sites and arrows indicating expected sites of phosphodiester bond cleavage. (B) Lanes 1 and 10: no enzyme. Lanes 2 and 3: AvaII 100/50 U. Lanes 4 and 5: AvrII 40/20 U. Lanes 6 and 7: BanI 20/10 U. Lanes 8 and 9: TaqI 40/20 U. (C) Additional assays using: different RNA:DNA ratio in substrate annealing reaction. Lanes 1–4: R:D ratio 1:1. Lanes 5–8, R:D ratio 0.5:1. Lanes 1 and 5: no enzyme. Lanes 2 and 6: AvaII 200 U. Lanes 3 and 7: AvrII 125 U. Lanes 4 and 8: BanI 200 U. Green bands, RNA; Red bands, DNA; Yellow bands, merged RNA and DNA. All assays were incubated for 180 min in NEBuffer 4 at 37°C with the exception of TaqI assay (65°C).
heteroduplex substrate at the same phosphodiester bonds that are hydrolyzed in an equivalent DNA–DNA homoduplex creating a 3-base 5′ extension. Fragment analysis data for the AvrII, BanI and TaqI products are presented in Supplementary Figure S1 and demonstrate that these enzymes also cleave RNA–DNA and DNA–DNA substrates at the same phosphodiester bonds.

RNA and DNA strands of heteroduplex substrates may be cleaved at different rates

Type IIP REases that act on palindromic or interrupted palindromic, DNA sequences are symmetric homodimeric enzymes that usually cleave both strands of a DNA duplex in a concerted fashion with the active site of each monomer cleaving the scissile bond on a single strand. REase hydrolysis of an RNA–DNA heteroduplex, however, might be expected to favor cleavage of one strand or the other due to the likely structural and chemical asymmetry of such a substrate. To investigate this we performed 60 min time-course analyses using the IM399R–IM398D heteroduplex and monitored the accumulation of the individual DNA and RNA products. The quantity of enzyme used in the assays was kept low to ensure that the substrate was not completely hydrolyzed during the period of measurement. Figure 4B shows the results of such analyses using AvaII (lanes 1–6) and BanI (lanes 7–12). In the case of AvaII both cleavage products are observed to accumulate at similar rates. Densitometric analyses using the unmerged FAM and TAMRA scan data indicate that 28% of the RNA and 36% of the DNA strand were hydrolyzed by the 60 min time-point (Figure 4B, lane 6). A very different result was obtained in the BanI assay where the enzyme exhibits a strong preference for hydrolysis of the DNA strand. In this case, 85% of the DNA was converted to product in 60 min (Figure 4B, lane 12) compared with only 13% cleavage of the RNA strand. Figure 4C shows the results of a similar time course assay using the same heteroduplex substrate and the REase HinfI. In this case, only the DNA strand appears to be susceptible to hydrolysis, with 34% of the substrate cleaved at the 60 min time point (Figure 4C, lane 6). Additional experiments using higher concentrations of HinfI (500–1500 U) indicate that limited hydrolysis of the DNA occurs but that cleavage never exceeds 10% of the total DNA strand in the assay (data not shown). Fragment analysis data for the HinfI RNA-cleavage product (and control RNA IM405R) are presented in Supplementary Figure S1 indicating that the same phosphodiester bond is hydrolyzed in the equivalent RNA (IM399R) and DNA (IM397D) strands in RNA–DNA and DNA–DNA duplex substrates, respectively.

Ribonucleotide substitution at variable positions within REase target sites can modulate cleavage activity on heteroduplex substrates

Oligoribonucleotide IM400R is the reverse-complementary sequence of IM399R (Table 1). An IM400R–IM397D heteroduplex therefore contains all the same restriction sites as IM399R–IM398D but with
the RNA and DNA strands reversed. The former, however, was observed to be a much poorer AvaII sub-
strate than the latter (data not shown). This could arise as a consequence of altered topology due to the strand
reversal or because IM400R contains uracil as the W
residue of the AvaII site (G/GWCC) where IM399R has
adenine. To address this, we constructed an IM431R–
IM432D heteroduplex (Table 1) that is identical to
IM399R–IM398D except that the former contains a
rU:dA base pair in the AvaII site where the latter
contains rA:dT. The results of time-course analyses of
AvaII cleavage of both substrates are shown in Figure 5.
As previously, the assay using the IM399R–IM398D sub-
strate (Figure 5, lanes 1–6) shows that the RNA and DNA
products accumulate at a similar rate. However, in the
IM431R–IM432D assay (Figure 5, lanes 7–12) the rate
of hydrolysis of the DNA strand appears to have been
reduced by a factor of \( \frac{1}{2} \) and there is little or no
evidence of RNA strand cleavage. Replacement of rA by
rU in the W position of the AvaII site therefore affects
cleavage of both strands of the substrate RNA–DNA
heteroduplex. A similar inhibitory effect upon RNA
strand cleavage is observed with HinfI (G/ANTC) when
the central position contains an rU:dA base pair (data not
shown).

Relative activities of REases on bacteriophage \( \lambda \) DNA,
DNA–DNA oligonucleotide duplex and RNA–DNA
heteroduplex substrates

The concentrations of BSA-free REase samples were
determined based on their absorbance at 280 nm (triplicate
assays) and the hypothetical extinction coefficients of the
individual enzymes calculated from their amino acid com-
position. Activities were measured on appropriately
diluted REases using bacteriophage \( \lambda \) DNA (1 \( \mu \)g = 0.03
pmol), IM397D–IM398D DNA duplex (1 pmol) and
IM399R–IM398D RNA–DNA heteroduplex (1 pmol) as
substrates. The 2-fold dilution series of enzymes were used
in end-point assays to identify the lowest concentration of
enzyme that resulted in complete (>95%) cleavage of each
substrate. Reaction products were resolved by agarose gel
electrophoresis in the case of bacteriophage \( \lambda \) DNA
and by denaturing-PAGE for oligonucleotide assays.
An example of the latter analysis is presented in Supplementary Figure S2 and the calculated activities of each REase are presented in Table 2. The activities of AvaII, AvrII, BanI, HinfI and TaqI (in pmol sites cleaved per λ unit) are essentially equivalent on both DNA substrates, varying by less than 3-fold between assays using λ DNA and the IM97D–IM398D oligonucleotide duplex.

AvaII, BanI, HinfI and TaqI REases each hydrolyze an RNA–DNA heteroduplex at least two orders of magnitude less efficiently than the equivalent DNA–DNA homoduplex. AvrII is much less active on duplex DNA than the other REases tested, hydrolyzing around 100 fmol of sites per λ unit compared to 1.6–3.2 pmol per unit for AvaII, BanI, HinfI and TaqI. However, unlike the other REases, AvrII cleaves homoduplex and heteroduplex substrates at near-equivalent rates, 0.1 and 0.033 pmol sites cleaved per λ unit, respectively (Table 2).

### Oligonucleotide-directed ‘restriction’ of long RNA molecules

Oligonucleotides IM433D–IM436D (Table 1) were used to construct an IVT template wherein a T7 promoter sequence was appended to a 175-bp DNA sequence–derived from part of the catG antibiotic resistance gene (27)–that was modified to introduce unique restriction sites for AvaII, AvrII, BanI, HinfI and TaqI REases. An IVT product was synthesized from this template, treated with alkaline phosphatase to remove the 5’-triphosphate group, 5’-phosphorylated with T4 polynucleotide kinase and attached to a FAM-labeled oligoribonucleotide (IM411R, Table 1) and T4 RNA Ligase 2 as described in the ‘Materials and Methods’ section. This yielded a population of RNA molecules, ~191-nt long, each containing a fluorescent reporter group at the 5’-end (Figure 6A). A duplex DNA substrate of identical sequence carrying a 5’-FAM-group was constructed via PCR amplification of the original IVT template PCR using oligonucleotides IM436 and IM448 (Table 1).

Oligonucleotides IM437D–IM440D and IM442D (Table 1) were designed to hybridize to the FAM-tagged RNA, in five separate annealing reactions, at positions that included the restriction sites for HinfI, AvaII, BanI, AvrII and TaqI REases, respectively. Restriction assays produced by restriction of a FAM-labeled DNA duplex with the cognate REase. Assays using HinfI (50 U), AvaII (500 U), BanI (100 U) and AvrII (10 U) were incubated for 60 min at 37°C. All five substrates were incubated in the absence (+) and presence (−) of the cognate REase. The TaqI assay (50 U) was incubated at 65°C for 60 min. Approximately 250 fmol of DNA were loaded in each gel lane. Size standards (M) were produced by restriction of a FAM-labeled DNA duplex with the same sequence as the RNA substrate and using the equivalent REases.

**Table 2.** Relative activities of restriction endonucleases on DNA–DNA and RNA–DNA substrates

| Endonuclease | AvaII | AvrII | BanI | HinfI | TaqI |
|--------------|-------|-------|------|-------|------|
| Specific Activity (units per mg) (µg λ DNA cleaved per hour per mg) | 2.6 × 10^6 | 1.9 × 10^5 | 5.3 × 10^5 | 3.7 × 10^5 | 2.5 × 10^6 |
| Cleavage sites in λ DNA (pmol λ sites cleaved per mg) | 35 | 2 | 25 | 148 | 121 |
| Ratio of DNA–DNA to RNA–DNA cleavage (pmol DNA–DNA oligo sites cleaved per unit) | 1.05 | 0.06 | 0.75 | 4.44 | 3.63 |
| Ratio of DNA–DNA to RNA–DNA cleavage (pmol RNA–DNA oligo sites cleaved per unit) | 0.0067 | 0.033 | 0.0042^b | 0.0036^c | 0.033 |

^1µg λ DNA = 0.03 pmol.

^2Based on RNA strand data (DNA strand of heteroduplex is cut at least an order of magnitude more rapidly).

^3Based on RNA strand data (No cleavage of DNA strand of heteroduplex was observed).
were carried out for all five enzymes using heteroduplex substrates where the annealed oligonucleotide spanned the cognate REase cleavage site. As previously, reaction products were resolved by denaturing polyacrylamide gel electrophoresis and FAM fluorescence was monitored using the Typhoon imager. Electrophoretic size standards were generated by cleavage of the 5′-FAM-labeled DNA duplex substrate with each of the five enzymes and mixing of the reaction products following dilution in denaturing loading buffer. Figure 6B shows the result of such an analysis and indicates that each REase cleaves the heteroduplex at a single site and that such cleavage does not occur in the absence of added endonuclease. The observed electrophoretic mobility of each restriction product is consistent with the expected sizes resulting from cleavage of the 191 nt RNA with HinfI (66 nt), AvaII (93 nt), BanI (105 nt), AvrII (120 nt) and TaqI (148 nt). In addition, the electrophoretic mobilities of the RNA cleavage products (Figure 6B, lanes 3, 5, 7, 9 and 11) appear to be essentially identical to those of the equivalent duplex DNA markers produced by restriction with the same enzymes (Figure 6B, lanes 1 and 12). Although slight size variation is observed for the restricted RNA products in each case, inspection of the equivalent uncut RNA samples suggests that this is a consequence of heterogeneity among the products of the IVT and/or splinted-ligation reactions rather than relaxed specificity of the relevant REases. We therefore conclude that these restriction enzymes are able to catalyze the site-specific hydrolysis of long-RNA molecules when directed to do so by hybridization of a short DNA oligonucleotide to the cognate cleavage site within the RNA.

Re-testing enzymes used by Molloy and Symons

Molloy and Symons reported that eight REases (AluI, EcoRI, HaeIII, HhaI, HindII, MspI, SalI and TaqI) could cleave one or both strands of an RNA–DNA heteroduplex substrate containing the relevant recognition sequences (15), whereas we only identified TaqI as having such activity in our experiments. We therefore re-tested each of these REases using IM482D–IM483D homoduplex and IM485R–IM483D heteroduplex substrates containing a single recognition site for each enzyme. Three hour assays using 100 U of each REase (Supplementary Figure S3) again confirms DNA and RNA-strand hydrolysis by TaqI (lane 18) and also reveals heteroduplex cleavage–of the RNA strand only–by HaeIII (lane 8).

DISCUSSION

By screening >200 different Type II REases we identified six enzymes that can cleave the RNA strand of an RNA–DNA heteroduplex substrate, in the form of synthetic oligonucleotide/oligoribonucleotide duplexes or via hybridization of DNA oligonucleotides to the relevant cognate sequences in longer RNA molecules prepared by IVT. Four of these hydrolyze both the RNA and DNA strands but, surprisingly, one of them (HinfI) only cuts the RNA strand well. The activities of HaeIII and TaqI on RNA–DNA hybrid substrates, described by Molloy and Symons in 1980 (15), are confirmed in the present study, but we could find no evidence of such activity for six additional candidate REases identified in their article. However, it is possible, even likely, that additional enzymes having low levels of such activity were missed during our initial screening experiments. Similarly, as these experiments all used reaction conditions optimized for cleavage of DNA by the different REases they might fail to detect an enzyme that was able to hydrolyze an RNA–DNA heteroduplex if assayed at a different pH or in the presence of different salts or divalent cations. In addition, it is well established that some REases require that two copies of their recognition sequence be bound simultaneously for them to become competent in catalysis (28). Clearly, such an enzyme would not be detectable using our screening system in the event that such binding were required to occur in cis. Despite such caveats, it seems that the capacity to hydrolyze RNA–DNA hybrids is the exception rather than the rule among Type II REases.

All the enzymes shown to cut the RNA strand of a heteroduplex substrate are members of the Type IIP subclass of REases, homodimeric enzymes that bind to, and cleave within, palindromic (AvrII, BanI, HaeIII and TaqI) or interrupted palindromic (AvaII, BanI and HinfI) DNA sequences, with each monomer catalyzing the concerted hydrolysis of one of the two DNA strands (6). With the exception of HaeIII, which has not been assigned to any of the five REase structural superfamilies, all these enzymes are members of the structural class that employ a canonical PD-(E/D)XK catalytic motif to achieve phosphodiester bond hydrolysis (5). In addition, five of the enzymes cleave the DNA duplex to generate hydrolysis products with a 5′-extension of 2 (TaqI), 3 (AvaII and HinfI) or 4 (AvaII and BanI) nt. However, the presence of the 5′-extensions and their lengths are likely less significant than the observation that this positional specificity is absolutely maintained when the substrate is not DNA but an RNA–DNA heteroduplex. This demonstrates that any structural changes that occur when one substrate DNA strand is replaced by RNA, for example transitioning to a more A-like conformation, are readily accommodated by the active sites of these REases. An intriguing possibility, that we hope to address by X-ray crystallographic studies, is that the enzymes that hydrolyze RNA–DNA heteroduplexes have evolved active site architectures that preferentially bind A-form DNA or induce structural transitions towards A-like conformations on DNA binding.

In addition to the likely role of topological differences in modulating cleavage rates for DNA–DNA and RNA–DNA substrates we also find evidence of sequence specific-effects. Most notably, while AvaII (recognition sequence C/CWGG where W = A or T) cleaves both strands of an RNA–DNA heteroduplex at more or less equivalent rates when the central base pair is rA:dT, hydrolysis of an otherwise identical substrate containing an rU:dA base pair is slightly impaired in DNA strand hydrolysis and cleavage of the RNA strand is no longer...
observed. As Ehrlich and co-workers have previously shown that AvaII efficiently cleaves *Bacillus subtilis* PBS1 bacteriophage DNA in which 98% of dT nucleotides are replaced by dU (29) it appears that the impairment we observe must arise from a combination of topological and base-specific effects. The same authors also show that PBSI DNA is restricted efficiently by TaqI (T/CGA) but that HinfI activity (G/ANTC) is significantly impaired when the dT residue in the fourth position on both strands is replaced by dU.

AvaII, BanI, HinfI andTaqI REases all restrict the RNA-strand of an RNA–DNA heteroduplex substrate at least two orders of magnitude less efficiently than the equivalent DNA–DNA homoduplex. In other respects, however, the properties of the different REases are quite distinct. AvaII and TaqI hydrolyze both strands of the IM399R–IM398D heteroduplex substrate at equivalent rates in what appears to be a concerted reaction similar to that expected for a Type IIP enzyme cleaving duplex DNA. The other two enzymes reveal strong and opposing, strand-specific biases, BanI preferentially hydrolyzing the DNA and HinfI favoring cleavage of the RNA strand, respectively. At present, we do not know what is responsible for the slow rate of heteroduplex cleavage by these enzymes. Nor do we know how AtrII REase is almost equally effective at cleaving sites in DNA and DNA–RNA hybrid substrates. Future studies, including structural and mechanistic analyses, will no doubt provide a deeper understanding of the properties of these unusually promiscuous restriction enzymes.

The capacity to cleave specific RNA phosphodiesters bonds using REases has potential applications in molecular biology. The 3′- and 5′-ends of RNA molecules—whether isolated from cells or synthesized by IVT—often show slight heterogeneity in length. The latter also contain modified 5′-termini, in the form of 5′-triphosphate and the 7-methylguanosine ‘cap’ in prokaryotes and eukaryotes, respectively. Although such RNAs can be ‘trimmed’ using ribozymes or DNAzymes to eliminate 3′-end heterogeneity (30), oligonucleotide-directed restriction can, in principle, be used to produce homogeneous RNAs with uniform 5′ and 3′ RNA termini. This is particularly applicable to RNAs produced by IVT where the relevant REase recognition sequences are readily introduced to the template DNA using PCR. A second possible application is in the construction of chimeric polynucleotides from multiple RNA (or RNA and DNA) molecules. As the REases cleave the RNA strand of an RNA–DNA heteroduplex at a specific phosphodiester bond it is relatively easy to construct such chimeras via splinted ligation reactions using T4 RNA ligase 2 (31). Oligonucleotide-directed RNA cleavage could also be exploited as a diagnostic tool for the identification of a particular RNA transcript (or viral RNA) within a complex RNA sample provided that the sequence of the target RNA is known and contains the relevant REase recognition sites. In principle, this could be combined with electrophoretic separation of the cleavage products to generate an RNA profile for an RNA virus or to identify alternatively spliced variants of eukaryotic mRNAs.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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