Identification of a non-junction phosphodiester that influences an autolytic processing reaction of RNA

Jamal M. Buzayan, Hans van Tol, Paul A. Feldstein and George Bruening*

Department of Plant Pathology, College of Agriculture and Environmental Sciences, University of California, Davis, CA 95616, USA

Received May 1, 1990; Revised and Accepted July 2, 1990

ABSTRACT

Oligoribonucleotides with specific sequences derived from the satellite RNA of tobacco ringspot virus undergo autolytic cleavage at the CpA phosphodiester that is the junction between unit sequences of multimeric satellite RNA. Buzayan et al. (*Nucleic Acids Res.*, 16, 4009–4023 (1988)) showed that an oligoribonucleotide with 97 satellite RNA-derived nucleotide residues self-cleaved with greatly reduced efficiency when it was synthesized *in vitro* from adenosine-5'-O-(1-thiotriphosphate) (abbreviated rATP α S) and three rNTPs. No other substitution of one rNTP by the corresponding rNTP α S had this effect, suggesting that a phosphorothioate CpA junction inhibits self-cleavage. Here, we replaced the usual CpA junction of a small self-cleaving oligoribonucleotide with a CpU junction. Self-cleavage of this molecule was reduced not only by rUTP α S-substitution, as expected, but also by partial and complete rATP α S-substitution. By analysis of the locations of rAMPS residues in cleavage products derived from partially rATP α S-substituted oligoribonucleotides, we identified A26 as the residue contributing the non-junction phosphorothioate diester that most strongly inhibited self-cleavage. Manganese ions strongly stimulated the self-cleavage of the rATP α S-substituted, CpU-junction oligoribonucleotide but was less effective when the junction was CpA.

INTRODUCTION

The polarity of the satellite RNA of tobacco ringspot virus that becomes encapsidated in tobacco ringspot coat protein (1) is designated as sTobRV(+)RNA. Both sTobRV(+)RNA and the complementary polarity sTobRV(–)RNA occur not only in the linear, 359 nucleotide residue (nt) 'monomeric' form but also as repetitive sequence multimers of this sequence (2,3). Naturally-occurring dimeric and trimeric sTobRV(+)RNA and *in vitro* transcripts from plasmids bearing circularly-permuted, dimeric satellite RNA sequences exhibited specific self-cleavage, that is, autolytic processing (4–6). The reaction generates a 2',3'-cyclophosphate and a 5'-hydroxyl as the new chain ends

(3–5). Autolytic processing is presumed to be an essential step in the replication of the satellite RNA (7,8).

A circularly permuted dimeric, or sub-dimeric, transcript may be represented in the form P_m-M-D_n, where M is an unpermuted, monomeric sTobRV RNA sequence and P and D are the promoter-proximal and promoter-distal segments of the transcript. Subscripts define numbers of nt derived from the sTobRV RNA sequence. Truncated sequences, with no full, unpermuted monomeric unit remaining, are represented by P_m-D_n. A P_m-D_n that retains sufficient sTobRV(+)RNA sequence (9,10) self-cleaves to P_n and D_m at the CpA phosphodiester that is the junction between unit sequences of multimers and conforms to a hammerhead (11,12) secondary structure model, as indicated in Fig. 1A.

An rNTP α S, i.e., a nucleoside-5'-O-(1-thiotriphosphate), has one non-bridging oxygen atom of the triphosphate replaced by a sulfur atom, introducing a chiral center. Bacteriophage T7 RNA polymerase (13), like other RNA polymerases (14), accepts the S_p diastereomer of an rNTP α S and catalyzes synthesis of phosphorothioate diesters of the R_p configuration. van Tol et al. (15) demonstrated, for both sTobRV(+)RNA (16) and sTobRV(–)RNA (17), that the R_p configuration junction phosphorothioate diester generates the endo-isomer of the 2',3'-cyclophosphorothioate product, as expected from a mechanism based on an in-line attack by the 2'-hydroxyl group (18).

Buzayan et al. (16) did not detect self-cleavage of the sTobRV(+)RNA P₁₁₇-M-D₂₄₇ at the end of a 1 hr transcription reaction with a substrate mixture of 97% rATP α S, 3% rATP, and three other rNTPs. P₁₁₇-M-D₂₄₇ with other single rNTP α S substitutions cleaved to the extent of 60% or more of the control value. rATP α S-substituted P₃₉-D₅₈ self-cleaved to the extent of 5% during a 1 hr transcription reaction, compared to 99% for the corresponding all phosphodiester molecule and each of the other three single rNTP α S-substituted forms of P₃₉-D₅₈. Even synthesis from rCTP, rGTP and rUTP resulted in a P₃₉-D₅₈ that self-cleaved to more than 90% of the control value. rATP α S-substituted P₁₅-D₅₂ cleaved to 18% of the control value; surprisingly, self-cleavage of rGTP α S-substituted P₁₅-D₅₂ was not detected. Buzayan et al. (16) obtained evidence of an effect of rGTP α S-substitution on the conformation of P₁₅-D₅₂ and

* To whom correspondence should be addressed

Figure 1. Phosphorothioate diesters derived from rATP α S and rUTP α S and their effects on self-cleavage of oligoribonucleotides containing sequences from sTobRV(+)RNA. (A) Oligoribonucleotide P₅-D₄₆ displayed in a hammerhead secondary structure. Upper case letters and numbering correspond to the wildtype sTobRV(+)RNA sequence (Buzayan et al., 1986Viro); lower case letters indicate nt derived from the vector. In the mutant sequence of P₅-D₄₆(U:A), U1 replaces A1, and A19 replaces U19. (B) Extent of self-cleavage of P₅-D₄₆ or P₅-D₄₆(U:A) (upper zones) to D₄₆ and D₄₆(U:A) (lower zones) at the end of a 1 hr transcription reaction. Each 50 μ l reaction mixture contained 3.8 μ Ci of [α -³²P]rCTP (Amersham). Transcripts were P₅-D₄₆ (lanes 1–3) and P₅-D₄₆(U:A) (lanes 4–6). Oligoribonucleotides were synthesized from rNTPs (lanes 1,4) or in reaction mixtures in which rATP α S replaced rATP (lanes 2,5) or rUTP α S replaced rUTP (lanes 3,6), as indicated by 'A' or 'U', respectively, under the lanes. Analysis was in an 8% polyacrylamide gel in 8 M urea, and detection was by autoradiography. Each reactant and product appears to be composed principally of two oligoribonucleotides, presumably because of inexact termination of transcription.

other oligoribonucleotides (17). They concluded that inhibited cleavage of rGTP α S-substituted P₁₅-D₅₂ does not reflect a strong or critical chemical interaction or even a specific steric effect, because in such event a pronounced reduction in self-cleavage of rGTP α S-substituted P₁₁₇-M-D₂₄₇ and P₃₉-D₅₈ should have been observed.

P₃₉-D₅₈ also was synthesized from a solution that contained rATP α S and tracer amounts of [³²P]rATP (16). After self-cleavage of [³²P]P₃₉-D₅₈ at the CpA junction, the enhanced accumulation of ³²P in the terminal cytidine-2',3'-cyclophosphate residue, compared to elsewhere in P₃₉, demonstrated the critical role of the phosphorothioate diester at the junction in retarding self-cleavage. However, this analysis did not eliminate possible effects on self-cleavage of rATP α S-derived phosphorothioate diesters at other locations.

MATERIALS AND METHODS

The oligodeoxyribonucleotides d(+)355*46(U:A) and d(–)46*1(U:A), respectively, dCTGTCTCCGGATGTGCTTTCCGGACTGATGAGTCCGTGAGGACGAAACAGGTC and dGACCTGTTTCGTCTCACGGACTCATCAGTCCGGAAA-GCATATCCGGA (Operon Technologies, Alameda, California), supplied a portion of a 3'-terminal restriction endonuclease Sal I site and the sequence of P₅-D₄₆(U:A) (Fig. 1A), except for the 5'-terminal guanylate residue. Oligodeoxyribonucleotides dT7(+) and dT7(–)359*355 (15) together provided the sequences of a bacteriophage T7 RNA polymerase promoter and P₅. We enzymically 5'-phosphorylated (19) dT7(–)359*355 and d(+)355*46(U:A). The two pairs of complementary molecules were hybridized (20), and the pairs were combined. Plasmid pIC19H (21), cut with restriction endonucleases Hinc II and EcoR I, and the insert were incubated with bacteriophage T4 DNA ligase (22), and the product was transformed into DH5 α *Escherichia coli* cells (23) to generate plasmid pT7(+)355*46(U:A). pT7+117/M/D (16, BamH I-linearized), pT7+39/58 (16, Hind III-linearized), pT7(+)355*46 (15), and pT7(+)355*46(U:A) (both Sal I-linearized) were the templates for the synthesis of P₁₁₇-M-D₂₄₇, P₃₉-D₅₈, P₅-D₄₆, and P₅-D₄₆(U:A) (Fig. 1A) respectively.

Typical transcription reactions were in 1 $\times$ transcription buffer

(40 mM Tris-HCl, pH 7.5, 20 mM NaCl) containing 6 mM MgCl₂, 2 mM spermidine-HCl, and 10 mM dithiothreitol. DNA template concentration was about 40 μ g/ml, and bacteriophage T7 RNA polymerase and RNasin were supplied at 4 U/ μ l and 0.8 U/ μ l, respectively. The sources of S_p rATP α S and S_p rGTP α S and the mixed S_p and R_p diastereomers of rCTP α S and rUTP α S were as before (16). The rNTPs, with or without replacement of one rNTP by the corresponding rNTP α S, were at 2.5 mM each, except that rCTP α S was supplied at 5 mM (16). The expected terminal sequences of the transcripts are as indicated (16; Fig. 1A).

Products of self-cleavage reactions were denatured by heating in formamide solution and analyzed by electrophoresis through polyacrylamide gel in 7 M or 8 M urea. The distribution of radioactivity in products was determined by Cerenkov counting of excised gel pieces or by two-dimensional scanning of β -particle emissions using an Ambis Radioanalytic Imaging System (16,17,24). 5'-end-labeling of D_n products and cleavage of the 5'-end-labeled oligoribonucleotides at phosphorothioate diesters by the action of iodoethanol were as described by Gish and Eckstein (25) and modified (17).

RESULTS

P₅-D₄₆ (15), with a CpA junction, self-cleaved efficiently in the all phosphodiester form and when synthesized in a reaction mixture in which rUTP α S (Fig. 1B) or rCTP α S (not shown) replaced the corresponding rNTPs. Self-cleavage of the rATP α S-substituted form (Fig. 1B, lane 2) was very limited, as expected, and was detected only on overexposed autoradiograms. P₅-D₄₆(U:A), with a CpU junction, also cleaved efficiently in the all phosphodiester (Fig. 1B, lane 4) and rCTP α S-substituted (not shown) forms. Both the junction-substituting replacement of rUTP by rUTP α S and replacement of rATP by rATP α S resulted in very limited self-cleavage of P₅-D₄₆(U:A) (Fig. 1B, lanes 5,6). rGTP α S-substituted molecules of both sequences remained uncleaved (data not shown), a non-specific effect already observed for P₁₅-D₅₂ (16).

S. Dahm and O.C. Uhlenbeck (personal communication; see also Mei et al., 1989 (18)) observed that manganese ions effectively stimulated the self-cleavage of hammerhead

Figure 2. Effects of magnesium ions, manganese ions, and spermidine-HCl on the extent of self-cleavage of phosphorothioate diester-containing P₅-D₄₆ and P₅-D₄₆(U:A). Oligoribonucleotides, as analyzed in Fig. 1B, lanes 2, 6 and 5, respectively, were of the wildtype sequence P₅-D₄₆ (A) and of the mutant sequence P₅-D₄₆(U:A) synthesized in reactions in which either rATPαS replaced rATP (A,C) or rUTPαS replaced rUTP (B). Each electrophoretically purified oligoribonucleotide was incubated for 2 hr at 0° (lane 1) or at 37° (lanes 2–8) in 1× transcription buffer containing 3 mM magnesium chloride, 3 mM manganese chloride, 2 mM spermidine-HCl, or combinations of these at the same final concentration, as indicated at the bottom of the figure. Analysis was in an 8% polyacrylamide gel in 8 M urea, and detection was by autoradiography and two-dimensional analysis of β-particle emission from the dried gel. The molar fraction of the total of reactant, P₅-D₄₆ or P₅-D₄₆(U:A) (upper zone), plus product, D₄₆ or D₄₆(U:A) (lower zone), that was found in the product zone was calculated, taking into account background radioactivity and relative cytidylate contents reactant and product. The fractions of product for the samples held on ice (for panels A, B, and C, lane 1, respectively, 0.015, 0.013, and 0.09) were subtracted from the fractions for the corresponding samples incubated at 37° to generate the fractional cleavage of the reactant during incubation, as listed below the lanes.

oligoribonucleotides into which phosphorothioate diesters had been incorporated at the junction. The three forms of phosphorothioate diester-containing P₅-D₄₆ and P₅-D₄₆(U:A) that failed to cleave to a significant extent during the transcription reaction (Fig. 1B) were electrophoretically purified. Each was incubated with manganese or magnesium ions or spermidine-HCl, or combinations of these. As is indicated by Fig. 2A, no combination of these ions supported more than about a 5% conversion of rATPαS-substituted P₅-D₄₆ to D₄₆. However, addition of manganese ions resulted in an extent of self-cleavage

that was greater than or equal to that observed for the relevant control (Fig. 2A, lane 3 versus 2, 5 versus 2, 6 versus 4, 8 versus 7). Similarly, the self-cleavage of rUTPαS-substituted P₅-D₄₆(U:A) generally was limited, exceeding 10% only when manganese ions were included in the incubation mixture (Fig. 2B, lanes 3,5,6,8). In contrast, P₅-D₄₆(U:A) synthesized from rATPαS in place of rATP (Fig. 2C) revealed a dramatic effect of manganese ions. 3 mM MnCl₂ in combination with MgCl₂ or spermidine-HCl, or both, resulted in more than 75% conversion of rATPαS-substituted P₅-D₄₆(U:A) to D₄₆(U:A) (Fig. 2C, lanes 5,6,8).

When P₅-D₄₆ and P₅-D₄₆(U:A) were synthesized in a solution containing a 1:1 molar ratio of rATPαS and rATP, and the other three rNTPs, about 17% of P₅-D₄₆ and 38% of P₅-D₄₆(U:A) self-cleaved during the 135 min transcription incubation. We considered the D₄₆ and D₄₆(U:A) recovered directly after the transcription reaction to have been released rapidly ('R' under lanes in Fig. 3). Subsequently, electrophoretically purified P₅-D₄₆ and P₅-D₄₆(U:A) were incubated in a solution containing 3 mM MnCl₂ to give additional, more slowly ('S' under lanes) generated D₄₆ and D₄₆(U:A). The conversion during the second incubation was about 50%. The products were 5'-end-labeled and treated with iodoethanol to reveal, by chain cleavage, the locations of phosphorothioate diesters (Fig. 3, lanes 1–4). Only one such phosphorothioate diester was significantly less well represented in the R preparations of D₄₆ or D₄₆(U:A) than in the S preparations: the one contributed by incorporation of rATPαS at position A26.

The comparison of phosphorothioate diester locations in R and S preparations also was performed with P₃₉-D₅₈ synthesized in a reaction mixture that contained equimolar amounts of rATP and rATPαS. Conversion of P₃₉-D₅₈ to D₅₈ was 20% during the 70 min transcription reaction and 50% during a subsequent 90 min incubation of electrophoretically purified P₃₉-D₅₈ in 2× transcription buffer containing 12 mM MgCl₂ and 4 mM spermidine-HCl. Fig. 3, lanes 5 and 6, shows that the A26-derived phosphorothioate diester was barely detected in the rapidly released mixture of products D₅₈ but was well represented in the more slowly released mixture of products of the S preparation (lane 6). The result in Fig. 3, lane 7 demonstrates that the cleavage products derived from the action of iodoethanol and not random degradation (lane 8). P₁₁₇-M-D₂₄₇ was synthesized from a 15:85 molar ratio of rATPαS:rATP, and the R and S preparations of products M and D₂₄₇ were analyzed for the locations of phosphorothioate diesters. The phosphorothioate diester derived from A26 was the only one found to be more well represented in the S than in the R preparations (data not shown).

DISCUSSION

Self-cleavage of oligoribonucleotide P₅-D₄₆(U:A), with the A1:U19 base pair of the wildtype sequence inverted (Fig. 1A), was expected because self-cleaving CpU junctions are known for both natural (11,26) and synthetic (e.g. 10,27,28) self-cleaving sequences of the hammerhead type. rUTPαS-derived phosphorothioate diesters had little or no effect on the self-cleavage of wildtype sTobRV(+)RNA sequences with CpA junctions; P₃₉-D₅₈ (16) and P₅-D₄₆ (Fig. 1B, lane 3) self-cleaved completely during the 1 hr transcription reaction, and P₁₁₇-M-D₂₄₇ (16) self-cleaved to 85% of the control, all-phosphodiester, value. Neglecting the substituted A:U base pair, these three

Figure 3. Distribution of phosphorothioate diesters derived from rATP α S in product from rapidly and slowly self-cleaving oligoribonucleotides. Oligoribonucleotides were synthesized from a reaction mixture that contained a 50:50 molar ratio of rATP α S and rATP. Transcripts were P₅-D₄₆ (lanes 1,2), P₅-D₄₆(U:A) (lanes 3,4), and P₃₉-D₅₈ (lanes 5-8). The transcription reaction mixture was incubated for 135 min (lanes 1-4) or 70 min (lanes 5-8), and the reactant P_m-D_n and the rapidly-generated (indicated by R under lanes) product D_n were recovered by preparative gel electrophoresis. P_m-D_n was incubated for 90 min in 1 $\times$ transcription buffer containing 3 mM manganese chloride (lanes 1-4) or 6 mM magnesium chloride, 2 mM spermidine-HCl (lanes 5-8), giving the slowly-generated (S under lanes) product D_n, which also was electrophoretically purified. The R and S preparations of D_n oligoribonucleotides were 5'-end labeled in a polynucleotide kinase-catalyzed reaction. The extent of substitution of phosphodiester by phosphorothioate diesters on the 5' sides of adenosine residues is indicated by the intensities of the corresponding zones after partial cleavage of the oligoribonucleotides by treatment with iodoethanol (25) (lanes 1-6). Aliquots of D₅₈ also were incubated without iodoethanol (lane 7) or with base (lane 8). Electrophoresis was in 20% polyacrylamide gel, 7 M urea. Unremoved marks, locating tracking dyes, appear in lane 4.

molecules have all of the uridyate residues of the mutant oligoribonucleotide P₅-D₄₆(U:A). Therefore, we attribute the inability of rUTP α S-substituted P₅-D₄₆(U:A) to self-cleave to the effects of a single phosphorothioate diester, that of the CpU junction.

Because the self-cleavage of P₃₉-D₅₈ was not detectably affected by phosphorothioate substitutions from any source except rATP α S, inhibitory effects on the self-cleavage of P₃₉-D₅₈ are

expected to be especially diagnostic of the contributions of specific rATP α S-derived phosphorothioate diesters. In fact, for all oligoribonucleotides tested, the A26-derived phosphorothioate was less well represented than any other rATP α S-derived phosphorothioate in the rapidly released product. The greater cleavage at A26 sites in lanes 1 and 3 (Fig. 3), compared to lane 5, probably was due, at least in part, to the longer time of incubation of the transcription reaction mixtures for the first two (Fig. 3 legend). The lack of adenylate residues in the P₅ portion of P₅-D₄₆ allows interpretation of the effects of rATP α S substitution in P₅-D₄₆ on the basis of the D₄₆ product alone.

The efficient self-cleavage of P₃₉-D₅₈ that had been synthesized from rGTP α S shows that even the phosphorothioate diester to the 3' side of A26, connecting A26 and G27, did not have an effect similar to placing a phosphorothioate diester to the 5' side of A26. A26 is the last adenylate of the sequence CUGAXGA, where X is A, C or U. CUGAXGA is conserved in known hammerhead structures (11,26). Whether the phosphorothioate diester contributed by A26 interferes with cleavage by a specific steric effect, or by failing to supply a catalytic function of which the phosphodiester is capable, is unknown. A conformation of a hammerhead structure computed on the bases of energy-minimization and molecular dynamics (18) would, in an analogous sTobRV(+)RNA structure, place the centers of the A26 phosphorothioate diester sulfur atom and the junction phosphorus atom 1.17 nm apart, a distance inconsistent with direct interaction of the A26 phosphodiester oxygen atom with atoms at or near the junction.

The efficacy of manganese ions in promoting the self-cleavage of phosphorothioate diester-containing oligoribonucleotides (S. Dahm and O.C. Uhlenbeck, personal communication) was here (Fig. 2) confirmed. Presumably, the relatively stronger coordination of manganese ions by phosphorus-bound sulfur than by phosphorus-bound oxygen (30), compared to the coordination of magnesium ions by these atoms, contributes to the observed manganese ion effect. The combination of magnesium and manganese ions and spermidine-HCl generally was more effective than any of the ions alone (Fig. 2B,C, lane 8). Whether this result is due to a synergistic effect of the three ions, or simply to the greater total concentration of divalent metal ions and spermidine-HCl, is unknown. rATP α S-substituted P₅-D₄₆(U:A), a molecule without phosphorothioate substitution at the junction, exhibited the strongest manganese ion effect. This result provides no evidence in favor of the action of a manganese ion, or a corresponding magnesium ion, as a specific catalyst acting at the bond that is to be cleaved.

An approach used in this work was to segregate a population of partially rATP α S-substituted molecules into those that cleaved rapidly and those that cleaved more slowly. Subsequently, we identified a phosphorothioate diester that is more poorly represented in the class of rapidly self-cleaving molecules than in the class of slowly-cleaving molecules. This approach should have applications to other nucleic acid molecules that can be separated on the basis of function, as has been accomplished by Waring (29), and could be used to identify critical nt modified in ways other than by phosphorothioate substitution.

ACKNOWLEDGMENTS

We appreciate the efforts of F. Eckstein, Max-Planck-Institut für Experimental Medizin, Göttingen, Federal Republic of Germany, who generously donated supplies of rGTP α S and rCTP α S, and

of H.-Y. Mei and T.C. Bruice, Department of Chemistry, University of California at Santa Barbara, who provided us with the coordinates of calculated hammerhead structures. We thank S. Dahm and O.C. Uhlenbeck of the Department of Chemistry and Biochemistry, University of Colorado, Boulder, for information on the effects of manganese ions on processing of phosphorothioate diester-containing oligoribonucleotides. This research was supported by the United States Public Health Service, under NIH grant GM37627, and by the Agricultural Experiment Station of the University of California.

REFERENCES

- Schneider, I.R. (1977) In *Beltsville Symposia in Agricultural Research. I. Virology in Agriculture*, Romberger, J.A., ed., Allenheld, Osmun & Co., Montclair, New Jersey, pp. 201–219.
- Kiefer, M.C., Daubert, S.D., Schneider, I.R., and Bruening, G. (1982) *Virology*, **137**, 371–381.
- Buzayan, J.M., Gerlach, W.L., Bruening, G., Keese, P., and Gould, A.R. (1986) *Virology*, **151**, 186–199.
- Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R., and Bruening, G. (1986) *Science*, **231**, 1577–1580.
- Buzayan, J.M., Gerlach, W.L., and Bruening, G. (1986) *Nature (London)*, **323**, 349–353.
- Gerlach, W.L., Buzayan, J.M., Schneider, I.R. and Bruening, G. (1986) *Virology*, **151**, 172–185.
- Symons, R.H., Haseloff, J., Visvader, J.E., Keese, P., Murphy, P.J., Gordon, K.H.J., and Bruening, G. (1985) In Maramorosch, K., and McKelvey, J.J., Jr. (eds.), *Subviral Pathogens of Plants and Animals: Viroids and Prions*. Academic Press, New York. pp. 235–263.
- Bruening, G., J.M. Buzayan, A. Hampel and W.L. Gerlach (1988) In Domingo, E., Holland, J., and Ahlquist, P. (eds.), *RNA Genetics, Retroviruses, Viroids, and RNA Recombination*. CRC Press Inc., Boca Raton, Florida. Vol.II, Chapter 6. pp. 127–145.
- Buzayan, J.M., Gerlach, W.L. and Bruening, G. (1986) *Proc. Nat. Acad. Sci. USA*, **83**, 8859–8862.
- Haseloff, J., and Gerlach, W.L. (1988) *Nature (London)* **334**, 585–591.
- Forster, A.C., and Symons, R.H. (1987) *Cell*, **49**, 211–220.
- Hutchins, C.J., Rathjen, P.D., Forster, A.C., and Symons, R.H. (1986) *Nucleic Acids Res.*, **14**, 3627–3640.
- Griffiths, A.D., Potter, B.V.L., and Eperon, I.C. (1987) *Nucleic Acids Res.*, **15**, 4145–4162.
- Eckstein, F. (1985) *Annu. Rev. Biochem.*, **54**, 367–402.
- van Tol, H., Buzayan, J.M., Feldstein, P.A., Eckstein, F., and Bruening, G. (1990) *Nucleic Acids Res.*, **18**, 1971–1975.
- Buzayan, J.M., P.A. Feldstein, C. Segrelles and G. Bruening (1988) *Nucleic Acids Res.*, **16**, 4009–4023.
- Buzayan, J.M., P.A. Feldstein, G. Bruening and F. Eckstein (1988) *Biochem. Biophys. Res. Commun.*, **156**, 340–347.
- Mei, H.-Y., Kaaret, T.W., and Bruice, T.C. (1989) *Proc. Nat. Acad. Sci. USA*, **86**, 9727–9731.
- England, T.E., Bruce, A.G., and Uhlenbeck, O.C. (1980) *Meth. Enzymol.*, **65**, 65–74.
- Zoller, M.Y., and Smith, M. (1983) *Methods Enzymol.*, **100**, 468–500.
- Marsh, J.L., Erfle, M., and Wykes, E.J. (1984) *Gene*, **32**, 481–485.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Seidman, C.E. (1987) In Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) *Current Protocols in Molecular Biology*. John Wiley and Sons, New York. pp. 1.8.1–1.8.3
- Feldstein, P.A., Buzayan, J.M., van Tol, H., deBear, J., Gough, G.R., Gilham, P. T., and Bruening, G. (1990) *Proc. Nat. Acad. Sci. USA*, **87**, 2623–2627.
- Gish, G., and Eckstein, F. (1988) *Science*, **240**, 1520–1522.
- Bruening, G. (1989) *Meth. Enzymol.*, **180**, 546–558.
- Ruffner, D. E., Dahm, S. C., and Uhlenbeck, O. C. (1989) *Gene*, **82**, 31–41.
- Sheldon, C. C., and Symons, R. H. (1989) *Nucleic Acids Res.*, **17**, 5679–5685.
- Waring, R.B. (1989) *Nucleic Acids Res.*, **17**, 10281–10293.
- Pecoraro, V.L., Hermes, J.D., and Cleland, W.W. (1984) *Biochemistry*, **23**, 5262–5271.