RNA (2001), *7*:622–631. Cambridge University Press. Printed in the USA.

METHOD

Evaluation of methylphosphonates as analogs for detecting phosphate contacts in RNA–protein complexes

DAGMAR DERTINGER and OLKE C. UHLENBECK

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215, USA

ABSTRACT

The well-studied interaction between the MS2 coat protein and its cognate hairpin was used to test the utility of the methylphosphonate linkage as a phosphate analog. A nitrocellulose filter binding assay was used to measure the change in binding affinity upon introduction of a single methylphosphonate stereoisomer at 13 different positions in the RNA hairpin. Comparing these data to the available crystal structure of the complex shows that all phosphates that are in proximity to the protein show a weaker binding affinity when substituted with a phosphorothioate and control positions show no change. However, in two cases, a methylphosphonate isomer either increased or decreased the binding affinity where no interaction can be detected in the crystal structure. It is possible that methylphosphonate substitutions at these positions affect the structure or flexibility of the hairpin. The utility of the methylphosphonate substitution is compared to phosphate ethylation and phosphorothioate substitution experiments previously performed on the same system.

Keywords: 3-ethyl-1-nitrosourea (ENU); deprotection; equilibrium binding constant; HPLC separation; MS2 bacteriophage; phosphate modification; phosphorothioate; RNA hairpin

INTRODUCTION

The ordered phosphodiester backbone of RNA often serves as an important feature for sequence-specific recognition by proteins. It is therefore desirable to have a biochemical method to accurately identify phosphates that directly interact with a protein. One approach uses a modification-interference protocol in which the RNA is reacted with 3-ethyl-1-nitrosourea (ENU) to prepare a mixture of RNAs containing on average a single ethylated phosphodiester linkage (Vlassov et al., 1980, 1981). Modified RNAs that bind to the protein are selected and the sites of modification identified by their lability at high pH. Phosphates whose alkylation is not observed among the bound RNAs correspond to positions where modification interferes with complex formation (Romby et al., 1985; Kjems et al., 1992). Although this method is convenient for locating regions of an RNA molecule that interact with the protein, comparison with available structural data suggests that it does not always accurately identify the precise sites of contact with individual phosphates.

A more successful strategy for locating protein–phosphate contacts has been to use RNAs containing phosphorothioate linkages where one of the nonbridging phosphate oxygens is replaced by sulfur (Milligan & Uhlenbeck, 1989; Schatz et al., 1991). RNA mixtures for modification-interference studies that contain solely the R_P-phosphorothioate can be obtained by in vitro transcription (Eckstein, 1985; Griffiths et al., 1987). If the RNA fragment is not too long, unique phosphorothioate linkages can also be introduced by chemical synthesis and the two stereoisomers separated by HPLC (Slim & Gait, 1991). Single phosphorothioate substitutions have been shown to perturb protein binding at discrete locations in numerous nucleic acid–protein complexes (Milligan & Uhlenbeck, 1989; Schatz et al., 1991; Lesser et al., 1992; Kurpiewski et al., 1996). In three cases where a comparison with available structural data could be made, phosphorothioates perturbed protein binding at virtually every site of a protein–phosphate contact and generally did not affect the binding affinity at sites where no contact was observed (Thorogood et al., 1996; Vörtler et al., 1998; Dertinger

Reprint requests to: Dr. Olke C. Uhlenbeck, Department of Chemistry and Biochemistry, 215 UCB, University of Colorado, Boulder, Colorado 80309-0215, USA; e-mail: Olke.Uhlenbeck@Colorado.edu.

et al., 2000). However, the magnitude of the change in free energy was usually quite small and the binding affinity was found to either increase or decrease in an unpredictable manner. In addition, in many cases, substitution of only one of the two nonbridging oxygens with sulfur affected binding of the protein and the effect did not necessarily correlate with the oxygens that participate in the contact observed in the crystal structure. The difficulty of detection and lack of predictability prompted a search for an alternative method for identifying protein–phosphate contacts.

The methylphosphonate linkage, where one of the two nonbridging oxygens is replaced by a methyl group, was used to identify phosphate contacts in DNA–protein complexes (Noble et al., 1984; Botfield & Weiss, 1994; Smith & McLaughlin, 1997). However, methylphosphonates have been used only rarely in studies of RNA–protein interactions, because the ribose methylphosphonate linkage is not chemically stable (Hamy et al., 1993; Pritchard et al., 1994). Although no enzymatic method exists for introduction of a methylphosphonate linkage, deoxynucleoside methylphosphonoamidites can be used to obtain a mixture of the two stereoisomers by chemical synthesis. This mixture can easily be separated by HPLC when the oligomer is short (Hamy et al., 1993; Lebedev et al., 1993; Pritchard et al., 1994). In contrast to the ethylated phosphate, the methylphosphonate modification is nearly isosteric with the phosphodiester linkage and therefore should not introduce any steric clashes with the protein or within the RNA. Unlike the phosphorothioate substitution, the methylphosphonate linkage is uncharged, which neutralizes a small section of the RNA backbone (Fig. 1A). This change in electrostatic potential can be expected to reduce the local water structure organization (Kulinska et al., 1997) as well as weaken the ionic attraction between RNA and protein. Therefore, the methylphosphonate linkage can be expected to be a useful phosphate analog, because the substitution of an oxygen by a methyl group should be drastic enough to disrupt protein–phosphate contacts and yet conservative enough to only locally disturb the RNA–protein interface.

In this work, we use the MS2 coat protein–operator complex (Witherell et al., 1991; Johansson et al., 1997; Peabody, 1997; Lago et al., 1998) to evaluate the thermodynamic consequences on protein binding caused by introduction of a single methylphosphonate linkage into the RNA hairpin. The MS2 system is well suited for three reasons. (1) The x-ray structure of the complex reveals eight protein–phosphate contacts of very diverse nature within a small RNA molecule (Valegård et al., 1997). (2) Experiments substituting single deoxynucleotide residues at 15 positions in the RNA revealed that only one of the 2′-hydroxyl groups contributed to the binding affinity of the RNA to the protein (Baidya & Uhlenbeck, 1995). This permits the use of the more chemically stable deoxyribose methylphosphonate linkages. (3) Experiments with both ethyl phosphotriester and phosphorothioate linkages have been completed in this system (Gott et al., 1993; Dertinger et al., 2000) so that data obtained for three types of phosphate modifications can be compared.

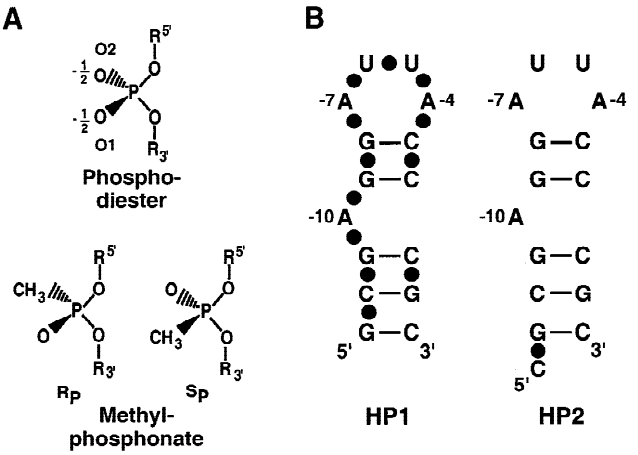

FIGURE 1. The methylphosphonate linkage and RNA hairpins. **A**: Chemical structures of phosphodiester and methylphosphonate linkages. According to standard rules of nomenclature (Cahn et al., 1966) replacement of the O1 oxygen with a methyl group, as defined by the crystal structure, yields the S_P methylphosphonate and substitution of O2 with a methyl groups leads to the R_P methylphosphonate (reverse from the assignment of phosphorothioate stereoisomers). **B**: RNA oligonucleotides used in this study are shown with the sites of methylphosphonate modifications denoted with black circles (●), with a deoxyribose nucleoside directly 5′ to the modified phosphodiester linkage. The numbering scheme of the RNA hairpin is based on the first nucleotide of the replicase gene (Witherell et al., 1991).

RESULTS AND DISCUSSION

The crystal structure of a 19-nt RNA hairpin bound to the MS2 coat protein homodimer (Valegård et al., 1997) reveals that, in addition to hydrogen bonds and stacking interactions with three bases and one 2′-hydroxyl group, contacts with the RNA phosphodiester backbone appear to play an important role in complex stabilization. If one conservatively defines primary protein–phosphate contacts as either hydrogen bonds that are less than 3 Å and in the correct geometry or ion pairs that are less than 4 Å length, then the cocrystal structure indicates that eight amino acid side chains interact with seven phosphates (Fig. 2). Three uncharged amino acids (TyrA85, AsnB55, and SerB52) serve as hydrogen bond donors and five charged amino acids (ArgA49, ArgB49, LysB61, LysB57, and LysA43) form ionic interactions with the phosphates. For three of the seven phosphates, only one of the two nonbridging oxygens participates in the contact, whereas both oxygens interact with the protein in the remaining four cases (Fig. 2B). These contacts vary from very simple interactions (i.e., the ion pair between LysA43 and O1 of phosphate −4)

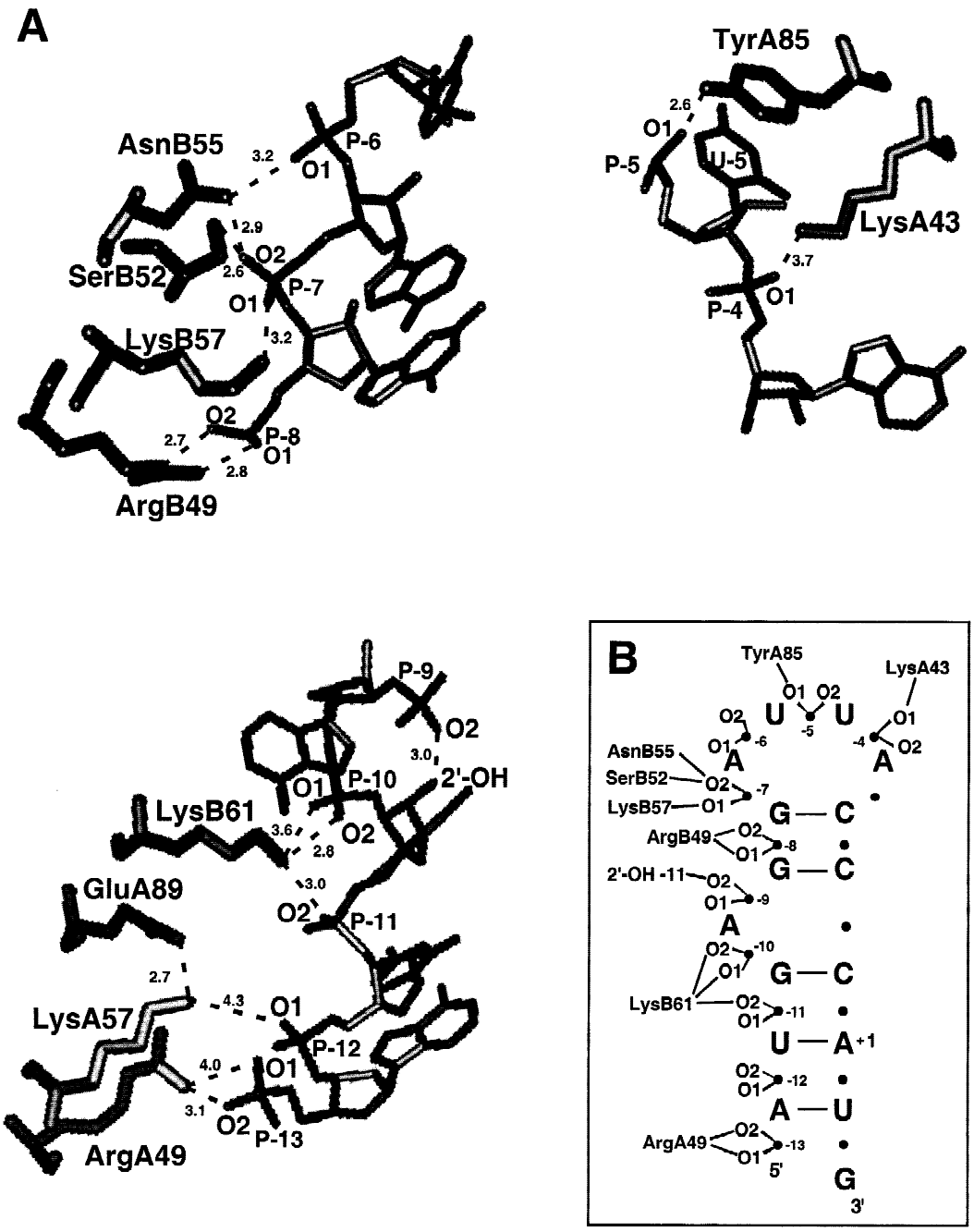

FIGURE 2. Protein–phosphate contacts as observed in the crystal structure of the MS2 coat protein–operator complex (Valegård et al., 1997). The prefixes A and B specify amino acids of the two protein subunits of the asymmetric coat protein dimer as defined in the crystal structure of the viral capsid (Valegård et al., 1990). **A**: Details of the interaction of the backbone phosphates with the protein. **B**: Sites of the primary protein–phosphate contacts, defined as either hydrogen bonds of less than 3 Å or ion pairs of less than 4 Å, and the intramolecular hydrogen bonds involving phosphate −9 are indicated on the secondary structure of the 19-nt RNA operator used in the crystallographic study. No electron density was observed for nucleotides −15, −14, and +4 (not shown). The protein binds the hairpin on one side, interacting with the loop and the 5′ half of the stem.

to complex networks of interactions involving several amino acids or phosphates (i.e., phosphate −7 interacting with LysB57, AsnB55, and SerB52 or phosphates −10 and −11 contacting LysB61). In addition to these primary contacts, the crystal structure suggests several other more distant interactions with phosphates that may also contribute to the overall binding affinity. LysA57 comes within 4.3 Å of phosphate −12, AsnB55 may form a hydrogen bond with O1 of phosphate −6 (Fig. 2A), and SerB51 is within 4 Å of both nonbridging oxygens of phosphate −7 (not shown). Finally, phosphate −9 forms an intramolecular hydrogen bond with

the 2′-hydroxyl group of nucleotide −11, which possibly stabilizes the structure of the RNA bound to the protein (Fig. 2A). One goal of this work is to use methylphosphonates to evaluate the relative thermodynamic contribution of each phosphate contact to the free energy of the complex.

Chemical synthesis was used to introduce individual deoxyribose methylphosphonate linkages into HP1 (Fig. 1B). This minimal RNA operator, when 5′-phosphorylated, can form all the contacts seen in the crystal structure and has previously been shown to bind to the protein with full affinity in vitro (Johansson et al., 1998; Dertinger et al., 2000). Because it was not possible to insert a methylphosphonate linkage at position −13 into HP1, a second oligomer, HP2, with an additional nucleotide at the 5′ end was used to study phosphate −13 (Fig. 1B). Single methylphosphonates were introduced at the seven phosphate positions of primary protein–phosphate contacts as well as six sites where no contacts are observed, but which are close to the RNA–protein interface. In each case, a deoxyribose nucleoside was introduced directly 5′ to the modified phosphate to improve chemical stability. This additional change is not expected to affect protein binding, because RNAs containing single deoxynucleotide substitutions had no detectable effect of K_d at most positions tested. The single exception is the deoxyribose at position −5, which is incorporated when phosphate −4 is substituted by a methylphosphonate (Baidya & Uhlenbeck, 1995). The small size of the RNA hairpins permitted excellent separation of the R_P and S_P isomers by reverse phase HPLC at every position except one (position −5), where only partial separation was achieved.

The affinities of MS2 coat protein for the unmodified HP1 and HP2, HP1 containing a deoxyribose at position −5, and the 26 methylphosphonate-containing RNAs were measured using a nitrocellulose filter binding assay. The data for the methylphosphonate-containing RNAs are presented in Table 1 as relative K_ds, which are obtained by dividing the K_ds of the corresponding control RNA by that of the modified RNA. In most cases, the all-RNA molecule could be used as a control because the deoxynucleotide had no thermodynamic effect. However, for the methylphosphonate at −4, the control RNA contained a deoxynucleotide at −5 to isolate the thermodynamic effect of the methylphosphonate. In general, the effects of single methylphosphonate modifications on binding are in a range that is convenient for detection and quantitation, with changes in free energy that lie between 0.4 kcal/mol and 4.0 kcal/mol. Of the 26 modified RNAs tested, 5 decreased the binding affinity by at least 20-fold compared to the corresponding control RNA, 12 bound between 2-fold and 10-fold weaker and 8 substitutions did not affect binding (less than 2-fold tighter or weaker). In only one case (R_P isomer at position −2) did replacement of a nonbridging oxygen with a methyl group slightly increase binding to the protein. At seven phosphate positions, substitution of both nonbridging oxygens decreased binding. At four other positions, replacement of only one of the two oxygens affected the binding affinity. No effect was observed for either isomer at the remaining positions tested.

To test whether the effect of a methylphosphonate modification is dependent upon buffer conditions, the relative binding of eight modified RNAs (R_P isomers at positions +1, −2, −3, −7, −9, and −11 and both stereoisomers at position −12) was tested under a variety of conditions, which differ from the standard buffer in either organic solvent content, pH, temperature, or ionic strength. Although molecular modeling and FTIR spectroscopic studies of dinucleoside methylphosphonates indicate little or no change in the sugar pucker and duplex structure (Swarna Latha & Yathindra, 1991; Kulinska et al., 1997), it is possible that a methylphosphonate linkage influences the local sugar pucker in the context of a structured RNA hairpin. As ethanol has been shown to favor A-form DNA (Ivanov et al., 1973; Ivanov & Krylov, 1992), the addition of ethanol may differentially affect the structure of methylphosphonate-containing RNAs. However, it was found that either 10% or 25% ethanol strengthens the complex between the protein and all RNAs to the same extent such that the relative equilibrium dissociation constants (K_ds) are comparable to those measured in the buffer without ethanol (data not shown). Because methylphosphonate substitutions are not expected to modify the pH or temperature dependence of the RNA structure, it is not surprising that there was no change in the relative K_ds when the pH was changed between 7.5 and 5.5, nor when the temperature was increased between 4 °C and 25 °C.

Finally, the affinities of the eight RNAs were also determined at both higher (0.62 M, 0.42 M, and 0.32 M) and lower (0.11 M) cation concentrations to see whether the removal of a charge in the RNA would change the ionic strength dependence of the interaction. Although the absolute K_ds increase with increasing ionic strength in a manner previously observed (Carey & Uhlenbeck, 1983; LeCuyer et al., 1996), the relative K_ds of the eight modified RNAs tested do not change (data not shown). It is surprising that there is no detectable difference in the ionic strength dependence of K_d. When ionic contacts in protein–RNA complexes are disrupted by protein mutagenesis, a change in the ionic strength dependence of K_d is generally observed (LeCuyer et al., 1996; GuhaThakurta & Draper, 2000). Because the methylphosphonate RNAs were chosen so as not to increase K_d too much, it is possible that the error in the experiments is too large to observe the 15–20% decrease in the slope of the log K_d versus log[M^+] plot expected for reducing the number of ion pairs by only one. Alternately, it is possible that disrupting an ionic contact from the protein side and the RNA side do not

TABLE 1. Effects of methylphosphonate modifications on K_d.

Position of phosphate[a]	Modified oxygen	Contacts of modified oxygen	Methylphosphonate: Relative K_d[b]	Phosphorothioate: Relative K_d[c]
+1	O1	none	*1.5*	*1.1*
	O2	none	*0.8*	*0.7*
−2	O1	none	*1.8*	*1.3*
	O2	none	**3.1**	*0.9*
−3	O1	none	*1.3*	*0.8*
	O2	none	**0.3**	*1.1*
−4	O1	LysA43	**0.5**	**2.7**
	O2	none	**0.5**	**2.4**
−5	O1	TyrA85	**0.02**	**0.3**
	O2	none	**0.02**	**0.5**
−6	O1	none	*1.0*	**14.5**
	O2	none	*0.6*	*1.8*
−7	O1	LysB57	**0.05**[d]	**3.9**
	O2	AsnB55, SerB52	**0.3**[d]	**0.3**
−8	O1	ArgB49	**0.002**	**0.3**
	O2	ArgB49	**0.003**	**4.4**
−9	O1	none	**0.5**	**0.5**
	O2	2′-OH-11	**0.1**	*0.8*
−10	O1	LysB61	**0.3**	**2.8**
	O2	LysB61	**0.2**	**0.2**
−11	O1	none	*0.9*	**0.5**
	O2	LysB61	**0.1**	*0.9*
−12	O1	none	**0.1**	*1.0*
	O2	none	**0.4**	*1.3*
−13	O1	ArgA49	*0.8*	*1.0*
	O2	ArgA49	**0.3**	**2.0**

[a]Modifications at all positions were introduced into HP1 with the exception of phosphate −13, which was modified in the background of HP2.

[b]Relative K_ds were obtained by dividing the value of the corresponding control RNA by that of the modified RNA. At every position except phosphate −4, the control was the all-RNA molecule because a deoxynucleotide 5′ to the phosphate had no effect on K_d (Baidya & Uhlenbeck, 1995). The control for phosphate −4 was a hairpin with a deoxynucleotide at −5. Effects that are less than twofold, shown in italics, are within the experimental error and therefore not regarded as significant.

[c]Data taken from Dertinger et al. (2000).

[d]The corresponding K_d values of the two methylphosphonate stereoisomers at position −7 have been switched previously (Johansson et al., 1998).

have the same consequences for the ionic strength dependence of K_d. When an ion pair forms between a protein and an RNA, a cation is displaced from the RNA and an anion is (often) displaced from the protein and they are jointly responsible for the ionic strength dependence of K_d. The methylphosphonate substitution may have only a relatively small effect on cation binding to the RNA hairpin because ion binding is, to a large degree, delocalized over the whole RNA molecule. In contrast, a protein mutation may often displace a specifically bound anion and thus reduce the ionic strength dependence of its binding to RNA. It will be interesting to mutate additional ionic contacts from both the protein and RNA sides and examine the consequences upon the ionic strength dependence of K_d.

In Figure 3A, the results of the methylphosphonate modification data are summarized on the hairpin secondary structure along with the positions of protein–phosphate contacts as observed in the crystal structure. All seven phosphates that directly interact with the protein show a decrease in binding affinity for at least one of the two methylphosphonate stereoisomers. Most of these effects are quite large, including the five modified RNAs with the largest changes in K_d of at least 20-fold. Even though a methylphosphonate replaces only one of the two nonbridging oxygens of a phosphate with a methyl group, one would generally assume that contacts formed by either oxygen can be weakened, because the methyl group cannot serve as a hydrogen bond acceptor and the remaining non-

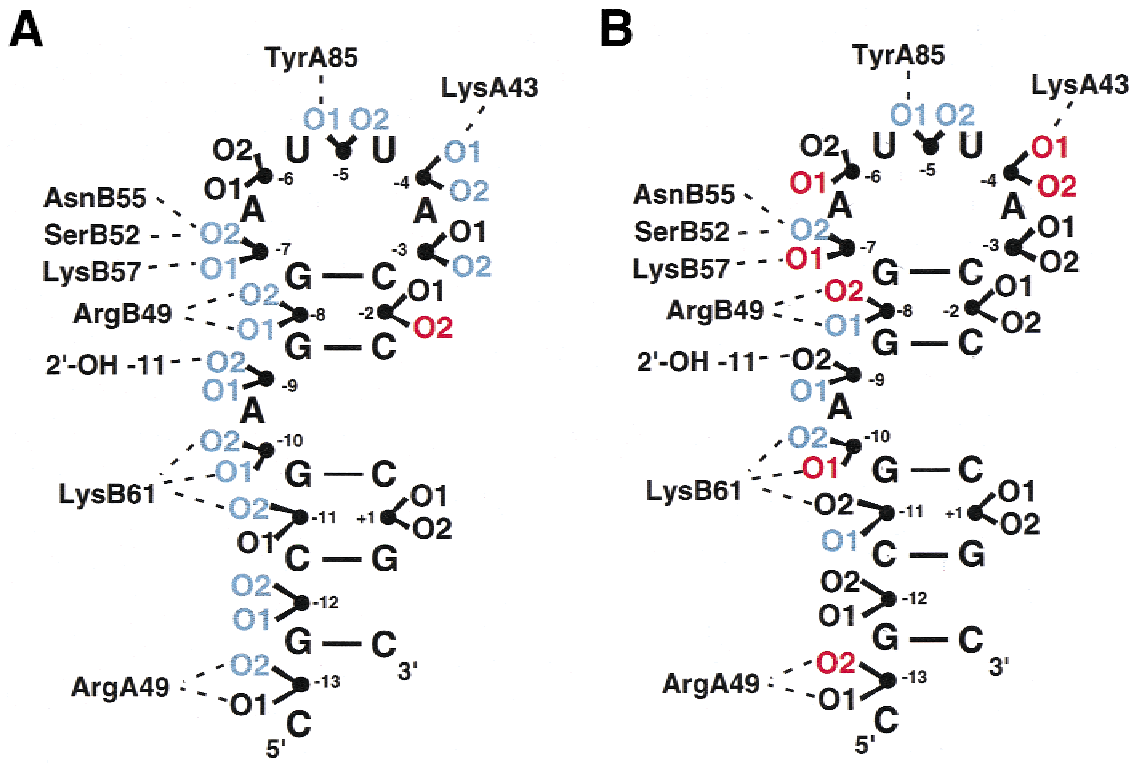

FIGURE 3. Changes in binding affinity due to phosphate modifications superimposed on the hairpin. All primary protein–phosphate contacts and the intramolecular hydrogen bond are indicated. Nonbridging oxygens where substitution increases the binding affinity are highlighted in red. Nonbridging oxygens where substitution weakens binding are shown in blue. Oxygens that can be substituted without a change in binding affinity are shown in black. **A**: Data obtained from the methylphosphonate modification study. **B**: Data taken from a previous phosphorothioate modification study (Dertinger et al., 2000).

bridging oxygen is no longer charged. It can therefore be expected that both methylphosphonate isomers impair binding whether or not one or both oxygens interact with the protein. For five of the seven phosphates that form direct contacts, both isomers affect the binding affinity as expected. However, in the case of phosphates −11 and −13, only one of the isomers interferes with complex formation. This may either be explained by an additional stabilizing hydrophobic interaction of the methyl group or the loss of an unfavorable effect caused by the negative charge of the phosphodiester linkage. In any case, the presence of a contact in the crystal structure always results in a decrease in binding when the corresponding phosphate is replaced by a methylphosphonate.

Of the six control phosphate sites where no contacts are predicted by the crystal structure, two show no affect on protein binding when either of the two oxygens is replaced by a methyl group. The first is position +1, which is on the opposite side of the RNA hairpin from the protein and therefore should be able to accommodate the modification. The second is phosphate −6, which is in the middle of the RNA–protein interface, but comes no closer than 3.2 Å to AsnB55. Although these two controls suggest that methylphosphonate substitutions can accurately identify protein–phosphate contacts, the fact that effects are seen at the remaining four control positions is less reassuring. However, in two of these cases, phosphates −9 and −12, reasonable explanations for the effects may be offered. The effects at position −12, which is within 4.3 Å of LysA57, may be caused by the disruption of a long-range electrostatic interaction between those residues (Fig. 2A). A similar observation was made for substrate binding of ribonuclease A, where basic amino acids that are more than 5 Å removed from the closest phosphate contribute to the overall binding affinity (Fisher et al., 1998). Phosphate −9 does not contact the protein, but forms a hydrogen bond with the 2′-hydroxyl of nucleoside −11 (Fig. 2A). This hydrogen bond that is disturbed by either methylphosphonate isomer may help to stabilize the structure of the bound RNA and therefore contribute to the overall binding affinity. However, it is difficult to explain the effects observed when the phosphodiester at position −2 or −3 is substituted with a methylphosphonate linkage. Both phosphates point out into solution away from the RNA–protein interface and should tolerate this replacement. Nevertheless, it cannot be excluded that the missing charge of the methylphosphonate at these two posi-

tions affects the structure or flexibility of the unbound RNA, thereby changing the free energy of the complex. It has been observed that introduction of several methylphosphonates on one side of a DNA helix causes bending of the nucleic acid by several degrees (Strauss-Soukup & Maher, 1997; Strauss-Soukup et al., 1997). The neutral methylphosphonate linkages are thought to change the repulsion between the phosphates in the backbone of a structured nucleic acid and could consequently alter the stability or flexibility of an RNA hairpin, leading to either an increase or decrease in the K_d of the RNA–protein complex.

Modification-interference experiments performed with ENU have shown that ethylation of all the phosphates between −15 and +1 of the MS2 hairpin interfere with MS2 coat protein binding (Gott et al., 1993). Although these experiments were not performed quantitatively and used a mixture of the two stereoisomers at each position, the results are in good agreement with the methylphosphonate substitution data presented here. Of the 13 positions that can be compared, only positions −6 and +1 disagree. Because ethylation of phosphate residues like the methylphosphonate substitution result in neutralization of an area of the RNA backbone, the similarity of the results is not surprising. However, the additional bulk of the ethyl group could potentially disrupt part of the RNA–protein interface due to steric clash. This may explain why phosphate −6 in the middle of the interface disturbs complex formation when ethylated, but not when substituted by a methylphosphonate. It may also account for the interference of ethylation at positions −14 and −15, which are close to the protein. These two phosphates are not well defined in the crystal structure and therefore were not tested by methylphosphonates modifications. Ethylation interference at position −1 and +1 is unexpected because these phosphates are far away from the protein. It is possible that, as in the case of phosphates −2 and −3, where both ethylation and methylphosphonate substitution decreased binding, alkylation of the phosphate changes the structure or flexibility of the unbound RNA hairpin and interferes with protein binding.

All 13 phosphates that were replaced by methylphosphonates in this study have also been substituted by the corresponding phosphorothioate isomers and their binding to the MS2 coat protein has been analyzed (Dertinger et al., 2000). Note that priority rules of nomenclature (Cahn et al., 1966) state that, in the case of the R_P methylphosphonate, the methyl group replaces O2 of the phosphodiester (Fig. 2A), whereas in the case of the R_P phosphorothioate, the sulfur replaces O1 of the phosphodiester. The relative K_ds of the phosphorothioate-containing RNAs are listed in Table 1 and the data is summarized on the hairpin in Fig. 3B. A comparison of the results obtained for the methylphosphonate and phosphorothioate substitutions reveals that both modifications indicate a similar subset of phosphates involved in protein–phosphate contacts. However, the effects of the methylphosphonate modification on binding are on average considerably greater than those caused by phosphorothioate linkages and nearly always reduce the binding affinity whereas phosphorothioates substitutions often result in tighter binding to the protein. These differences can be understood in terms of the different chemical properties of the two modifications. The neutral methylphosphonate linkage is more effective in disrupting a given interaction than the corresponding phosphorothioate substitution. There are, however, some differences in the two data sets that are more difficult to explain. The sensitivity of phosphate −12 to replacement by a methylphosphonate and not a phosphorothioate is possibly due to the greater change of the electrostatic potential caused by the methylphosphonate, which may disrupt long-range charge–charge interactions providing part of the overall free energy of binding. Phosphates −2 and −3 tolerate a sulfur at either O1 or O2, whereas the R_P methylphosphonate isomers at both positions affect binding. This may indicate the larger sensitivity of methylphosphonate modification studies, but could also be due to a more severe change in the properties of the unbound RNA due to the substitution. Phosphate −6 is the only position where a phosphorothioate isomer affects binding, whereas binding is not affected by either of the methylphosphonate isomers. This curious result possibly reflects a unique effect that the phosphorothioate linkage may have on the rearrangement of the RNA structure between the bound and the free form (Kerwood & Borer, 1996; Valegård et al., 1997; Smith & Nikonowicz, 2000).

The data presented here can be compared with a similar study performed on the interaction of HIV-1 rev protein interacting with its RRE hairpin target (Pritchard et al., 1994). Of the eight phosphate positions substituted with methylphosphonates, four (positions 103, 104, 124, and 125) showed substantial reduction in the binding affinity for both isomers, one (position 106) only showed weaker binding with the R_P, and three (positions 105, 133, and 135) showed no effect with either isomer. These data are in good agreement with a structure of a rev peptide–RRE complex that subsequently appeared (Battiste et al., 1996). The four phosphates where both methylphosphonate isomers weaken the binding of rev are close to three arginines and one threonine, whereas the three positions where no effect is observed upon substitution are not close to the protein. However, phosphate 106, where a methyl group at 02 disrupts binding, is not in proximity to the protein. Because phosphate 106 lies in the internal bulge that causes a widening of the major groove that allows peptide binding, it is possible that the methylphosphonate modification exerts its effect by disrupting the RNA structure in this region. It is interesting that ENU modification–interference experiments done on the rev–RRE system

(Kjems et al., 1992) correctly identified all the phosphate sites found by methylphosphonate substitutions, but failed to identify several protein phosphate contacts and incorrectly implicated one phosphate as a site of contact. Thus, a reevaluation of the available data on the rev–RRE system suggest that the use of methylphosphonates can accurately identify RNA–protein contacts predicted by a structure.

From this work, it can be concluded that deoxyribose methylphosphonate linkages are well suited for studying protein–phosphate contacts in a sequence-specific RNA–protein complex, provided that the effect caused by the absence of the corresponding 2′-hydroxyl group can be controlled for. In contrast to the ENU modification, the methylphosphonate linkage does not introduce extra steric bulk and therefore identifies phosphates that are involved in specific interactions with higher accuracy. Although the phosphorothioate linkage predicts protein–phosphate contacts seen in the crystal structure somewhat more reliably, the changes in binding affinity are very small and therefore, require a very accurate assay for determining K_d. Because the changes in binding affinity caused by methylphosphonate substitutions are generally much larger, they are more easily detected. However, the neutral methylphosphonate linkage is more likely to disrupt RNA structure than the phosphorothioate, so occasional "false positive" sites are obtained. An additional advantage of the methylphosphonate linkage is that it nearly always decreases the binding affinity, whereas phosphorothioate modifications can either strengthen or weaken the RNA–protein complex. Therefore, in the case of the methylphosphonate linkage, the tedious separation of the two stereoisomers by HPLC may not generally be necessary for the identification of phosphates contacting the protein. This latter advantage makes the methylphosphonate linkage the most useful phosphate analog currently available.

MATERIALS AND METHODS

RNA synthesis

HP1 and HP2 were synthesized on a 1 μmol scale by standard phosphoramidite chemistry (Usman et al., 1987) using an ABI DNA/RNA synthesizer. Deoxyribose methylphosphonate linkages were incorporated into the RNA at specific positions by the use of the corresponding 2′-deoxynucleoside methylphosphonoamidites. This results in an RNA oligonucleotide with one deoxyribose directly 5′ to the methylphosphotriester. Solid supports, ancillary reagents, RNA amidites and 2′-deoxynucleoside methylphosphonoamidites were purchased from Glen Research.

Unmodified HP1 and HP2 as well as HP1 containing a deoxyribose at position −5 were deprotected as described previously (Wincott et al., 1995). After complete deprotection, these RNA samples were purified by electrophoresis on a denaturing 20% polyacrylamide gel. In the case of the methylphosphonate-containing RNAs, the base lability of the deoxyribose methylphosphonate required several changes in the deprotection protocol to minimize losses. Deprotection was attempted in several different ways and the best yield was obtained with the following protocol. To cleave oligonucleotides from the controlled pore glass beads and remove the base protecting groups, the RNA was incubated in 500 μL of a freshly made ethanol (100%)/acetonitrile/ammonium hydroxide (15 M) mixture (45:45:10) for 30 min at room temperature. Then, an equal volume of ethylenediamine was added and the RNA was incubated for 6 h at room temperature. The 1-mL deprotection reaction was desalted using a NAP25 size exclusion column (2.5 mL maximum sample volume; Pharmacia) with 50 mM TrisCl (pH 7.5)/10% acetonitrile as the equilibration buffer. The samples were dried using a speed-vac. Removal of the 2′-hydroxyl protecting groups was achieved as described previously (Wincott et al., 1995). The methylphosphonate-containing RNAs were then directly purified by HPLC. Despite these precautions, the overall yield was still quite low (approximately 20% of the starting material).

The two methylphosphonate stereoisomers were separated by HPLC on a Nucleosil reverse phase column (Alltech). The best resolution was achieved at 65 °C and a flow rate of 1.5 mL/min in a 0.1 M ammonium acetate buffer system (pH 8.0). Increasing the acetonitrile concentration from 5.6% to 7.4% allowed full separation of the R_P and S_P isomers at all positions but one. The peaks of the two stereoisomers at position −5 overlapped to a large extent and therefore only partial separation was achieved. In each case, the early-eluting isomer is assumed to correspond to the R_P isomer and the late-eluting isomer to the S_P isomer (Hamy et al., 1993; Lebedev et al., 1993; Pritchard et al., 1994).

RNAs were labeled at their 5′ end with T4 polynucleotide kinase and [γ^{32}P]-ATP. RNAs with a modified phosphate at position −12 or −13 could not be labeled with T4 polynucleotide kinase because the enzyme specifically interacts with the phosphate closest to the 5′ end and does not recognize the methylphosphonate linkage. These hairpins and the corresponding control RNAs, HP1 and HP2, were 3′-labeled with 5′[^{32}P]-pCp and T4 RNA ligase (England & Uhlenbeck, 1978). The labeled hairpins were subsequently purified by electrophoresis on a denaturing 20% polyacrylamide gel. Partial alkaline hydrolysis was used to identify the presence and correct position of the methylphosphonate linkage as well as the absence of the corresponding 2′-hydroxyl group (Johansson et al., 1998).

Equilibrium dissociation constants

All binding experiments were performed using a double mutant of the MS2 coat protein (V75E/A81G), which forms stable dimers in solution, but can no longer assemble into viral capsids. This protein recognizes the RNA hairpin in the same manner as the wild-type protein (LeCuyer et al., 1995). The protein was overexpressed in *Escherichia coli* BL21 pLysS FB810 cells (Benson et al., 1994) and purified as described previously (LeCuyer et al., 1996).

A nitrocellulose filter binding assay in a microtiter format was used to determine the K_d of all-RNAs (Johansson et al., 1998) using a buffer containing 10 mM $MgCl_2$, 80 mM KCl, and 100 mM HEPES (pH 7.5) unless noted otherwise. The variation of absolute K_d values of one given hairpin was less

than threefold in independent experiments. Each experiment included an unmodified control RNA to account for differences in protein preparation and experimental variability of the serial protein dilutions. Unmodified all-ribose HP1 was used as the control RNA for hairpins containing a deoxyribose methylphosphonate linkage at positions +1, −2, −3, −5, −6, −7, −8, −9, −10, −11, or −12, because the RNAs containing the corresponding deoxyribose substitution bind to the protein with the same affinity as the all-ribose hairpin (Baidya & Uhlenbeck, 1995). HP1 with a methylphosphonate linkage at position −4 lacks the 2′-hydroxyl group of nucleoside −5 that is required for high affinity binding. Therefore, the binding affinity of this modified hairpin was compared to the binding affinity of HP1 with a deoxyribose at position −5, which binds the protein 120-fold more weakly than the all-ribose HP1. The methylphosphonate linkage at position −13 was incorporated into HP2 and therefore, all-ribose HP2 was used as the control RNA. Relative binding affinities were calculated by dividing the K_d value of the unmodified RNA by that of the modified RNA determined in the same experiment. Each experiment was repeated at least three times and individual results are within twofold of the reported average.

ACKNOWLEDGMENTS

We are grateful to L.S. Behlen for the synthesis of all-RNA oligonucleotides and D. Wuttke and members of the Uhlenbeck lab for stimulating discussions and comments on the manuscript. D.D. would like to thank R. Seckler for helpful advice and continuing support. This work was supported by National Institutes of Health Grant GM36944 to O.C.U. and graduate student fellowships from the Gottlieb Daimler- and Karl Benz-Foundation and the German Academic Exchange Service (DAAD) to D.D.

Received October 30, 2000; returned for revision November 22, 2000; revised manuscript received January 17, 2001

REFERENCES

Baidya N, Uhlenbeck CO. 1995. The role of 2′-hydroxyl groups in an RNA–protein interaction. *Biochemistry 34*:12363–12368.

Battiste JL, Mao H, Rao NS, Tan R, Muhandiram DR, Kay LE, Frankel AD, Williamson JR. 1996. Alpha helix-RNA major groove recognition in an HIV-1 rev peptide-RRE RNA complex. *Science 273*: 1547–1551.

Benson FE, Stasiak A, West SC. 1994. Purification and characterization of the human Rad51 protein, an analogue of *E. coli* RecA. *EMBO J 13*:5764–5771.

Botfield MC, Weiss MA. 1994. Bipartite DNA recognition by the human Oct-2 POU domain: POU_s-specific phosphate contacts are analogous to those of bacteriophage lambda repressor. *Biochemistry 33*:2349–2355.

Cahn RS, Ingold SC, Prelog V. 1966. Specification of molecular chirality. *Angew Chem Internat Edit 5*:385–415.

Carey JC, Uhlenbeck OC. 1983. Kinetic and thermodynamic characterization of the R17 coat protein–RNA interaction. *Biochemistry 22*:2610–2615.

Dertinger D, Behlen LS, Uhlenbeck OC. 2000. Using phosphorothioate-substituted RNA to investigate the thermodynamic role of phosphates in a sequence-specific RNA–protein complex. *Biochemistry 39*:55–63.

Eckstein F. 1985. Nucleoside phosphorothioates. *Annu Rev Biochem 54*:367–402.

England T, Uhlenbeck OC. 1978. 3′-terminal labelling of RNA with T4 RNA ligase. *Nature 275*:560–561.

Fisher BM, Ha JH, Raines RT. 1998. Coulombic forces in protein–RNA interactions: Binding and cleavage by ribonuclease A and variants at Lys7, Arg10, and Lys66. *Biochemistry 37*:12121–12132.

Gott JM, Pan T, LeCuyer KA, Uhlenbeck OC. 1993. Using circular permutation analysis to redefine the R17 coat protein binding site. *Biochemistry 32*:13399–13404.

Griffiths A, Potter B, Eperon I. 1987. Stereospecificity of nucleases towards phosphorothioate-substituted RNA: Stereochemistry of transcription by T7 RNA polymerase. *Nucleic Acids Res 15*:4145–4162.

GuhaThakurta D, Draper DE. 2000. Contributions of basic residues to ribosomal protein L11 recognition of RNA. *J Mol Biol 295*:569–589.

Hamy F, Asseline U, Grasby J, Iwai S, Pritchard C, Slim G, Bulter PJG, Karn J, Gait MJ. 1993. Hydrogen-bonding contacts in the major groove are required for human immunodeficiency virus type-1 *tat* protein recognition of TAR RNA. *J Mol Biol 230*:111–123.

Ivanov VI, Krylov DY. 1992. A-DNA in solution as studied by diverse approaches. *Methods Enzymol 211*:111–127.

Ivanov VI, Minchenkova LE, Schyolkina AK, Poletayev AI. 1973. Different conformations of double-stranded nucleic acid in solution as revealed by circular dichroism. *Biopolymers 12*:89–110.

Johansson HE, Dertinger D, LeCuyer KA, Behlen LS, Greef CH, Uhlenbeck OC. 1998. A thermodynamic analysis of the sequence specific binding of RNA by MS2 coat protein. *Proc Natl Acad Sci USA 95*:9244–9249.

Johansson HE, Liljas L, Uhlenbeck OC. 1997. RNA recognition by the MS2 phage coat protein. *Semin Virol 8*:176–185.

Kerwood DJ, Borer PN. 1996. Structure refinement for a 24-nucleotide RNA hairpin. *Magn Res Chem 34*:S136–S146.

Kjems J, Calnan BJ, Frankel AD, Sharp PA. 1992. Specific binding of a basic peptide from HIV-1 Rev. *EMBO J 11*:1119–1129.

Kulinska K, Sarzynska J, Szabo T, Stawinski J. 1997. FTIR study on nucleotide analogues. 1. Spectral characterization of dinucleoside methylphosphonates and dinucleoside 5′-methylenephosphonates in solution and in solid phase. *J Biomol Struct Dyn 15*:119–128.

Kurpiewski MR, Koziolkiewicz M, Wilk A, Stec WJ, Jen-Jacobson L. 1996. Chiral phosphorothioates as probes of protein interactions with individual DNA phosphoryl oxygens: Essential interactions of *Eco*RI endonuclease with the phosphate at pGAATTC. *Biochemistry 35*:8846–8854.

Lago H, Fonseca SA, Murray JB, Stonehouse NJ, Stockley PG. 1998. Dissecting the key recognition features of the MS2 bacteriophage translational repression complex. *Nucleic Acids Res 26*:1337–1344.

Lebedev AV, Frauendorf A, Vyazovkina EV, Engels JW. 1993. Determination and prediction of the absolute configuration of dinucleoside alkylphosphonates using conformational analysis and multivariate statistics. *Tetrahedron 49*:1043–1052.

LeCuyer KA, Behlen LS, Uhlenbeck OC. 1995. Mutants of the bacteriophage MS2 coat protein which alter its cooperative binding to RNA. *Biochemistry 34*:10600–10606.

LeCuyer KA, Behlen LS, Uhlenbeck OC. 1996. Mutagenesis of a stacking contact in the MS2 coat protein-RNA complex. *EMBO J 15*:6847–6853.

Lesser DR, Grajkowski A, Kurpiewski MR, Koziolkiewicz M, Stec WJ, Jen-Jacobson L. 1992. Stereoselective interaction with chiral phosphorothioates at the central DNA kink of the *Eco*RI endonuclease–GAATTC complex. *J Biol Chem 267*:24810–24818.

Milligan JF, Uhlenbeck OC. 1989. Determination of RNA–protein contacts using thiophosphate substitutions. *Biochemistry 28*:2849–2855.

Noble SA, Fisher EF, Caruthers MH. 1984. Methylphosphonates as probes of protein–nucleic acid interactions. *Nucleic Acids Res 12*:3387–3404.

Peabody DS. 1997. Role of the coat protein–RNA interaction in the life cycle of bacteriophage MS2. *Mol Gen Genet 254*:358–364.

Pritchard CE, Grasby JA, Hamy F, Zacharek AM, Singh M, Karn J, Gait MJ. 1994. Methylphosphonate mapping of phosphate contacts critical for RNA recognition by the human immunodeficiency virus tat and rev proteins. *Nucleic Acids Res 22*:2592–2600.

Romby P, Moras D, Bergdoll M, Dumas P, Vlassov VV, Westhof E, Ebel JP, Giege R. 1985. Yeast tRNAAsp tertiary structure in solution and areas of interaction of the tRNA with aspartyl-tRNA synthetase. A comparative study of the yeast phenylalanine system by phosphate alkylation experiments with ethylnitrosourea. *J Mol Biol 184*:455–471.

Schatz D, Leberman R, Eckstein F. 1991. Interaction of *Escherichia coli* $tRNA^{Ser}$ with its cognate aminoacyl-tRNA synthetase as determined by footprinting with phosphorothioate-containing tRNA transcripts. *Proc Natl Acad Sci USA 88*:6132–6136.

Slim G, Gait MJ. 1991. Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. *Nucleic Acids Res 19*:1183–1188.

Smith JS, Nikonowicz EP. 2000. Phosphorothioate substitution can substantially alter RNA conformation. *Biochemistry 39*:5642–5652.

Smith SA, McLaughlin LW. 1997. Probing contacts to the DNA backbone in the trp repressor-operator sequence-specific protein–nucleic acid complex using diastereomeric methylphosphonate analogues. *Biochemistry 36*:6046–6058.

Strauss-Soukup JK, Maher LJ 3rd. 1997. Role of asymmetric phosphate neutralization in DNA bending by PU.1. *J Biol Chem 272*:31570–31575.

Strauss-Soukup JK, Vaghefi MM, Hogrefe RI, Maher LJ 3rd. 1997. Effects of neutralization pattern and stereochemistry on DNA bending by methylphosphonate substitutions. *Biochemistry 36*:8692–8698.

Swarna Latha Y, Yathindra N. 1991. Molecular mechanics studies of dinucleoside methylphosphonates: Influence of methylphosphonate and its chirality on the phosphodiester conformation. *J Biomol Struct Dyn 9*:613–631.

Thorogood H, Grasby JA, Connolly BA. 1996. Influence of the phosphate backbone on the recognition and hydrolysis of DNA by the *Eco*RV restriction endonuclease. A study using oligodeoxynucleotide phosphorothioates. *J Biol Chem 271*:8855–8862.

Usman N, Ogilvie KK, Jiang M-Y, Cedergren RJ. 1987. Automated chemical synthesis of long oligoribonucleotides using 2′-O-silylated ribonucleoside 3′-*O*-phosphoramidites on a controlled-pore glass support: Synthesis of a 43-nucleotide sequence similar to the 3′-half molecule of an *Escherichia coli* formylmethionine tRNA. *J Am Chem Soc 109*:7845–7854.

Valegård K, Liljas L, Fridborg K, Unge T. 1990. The three-dimensional structure of the bacterial virus MS2. *Nature 345*:36–41.

Valegård K, Murray JB, Stonehouse NJ, van den Worm S, Stockley PG, Liljas L. 1997. The three-dimensional structures of two complexes between recombinant MS2 capsids and RNA operator fragments reveal sequence-specific protein–RNA interactions. *J Mol Biol 270*:724–738.

Vlassov VV, Giege R, Ebel JP. 1980. The tertiary structure of yeast tRNAPhe in solution studied by phosphodiester bond modification with ethylnitrosourea. *FEBS Lett 120*:12–16.

Vlassov VV, Giege R, Ebel JP. 1981. Tertiary structure of tRNAs in solution monitored by phosphodiester modification with ethylnitrosourea. *Eur J Biochem 119*:51–59.

Vörtler CS, Fedorova O, Persson T, Kutzke U, Eckstein F. 1998. Determination of 2′-hydroxyl and phosphate groups important for aminoacylation of *Escherichia coli* $tRNA^{Asp}$: A nucleotide analogue interference study. *RNA 4*:1444–1454.

Wincott F, DiRenzo A, Shaffer C, Grimm S, Tracz D, Workman C, Sweedle D, Gonzalez C, Scaringe S, Usman N. 1995. Synthesis, deprotection, analysis and purification of RNA and ribozymes. *Nucleic Acids Res 23*:2677–2684.

Witherell GW, Gott JM, Uhlenbeck OC. 1991. Specific interaction between RNA phage coat proteins and RNA. *Prog Nucleic Acid Res Mol Biol 40*:185–220.