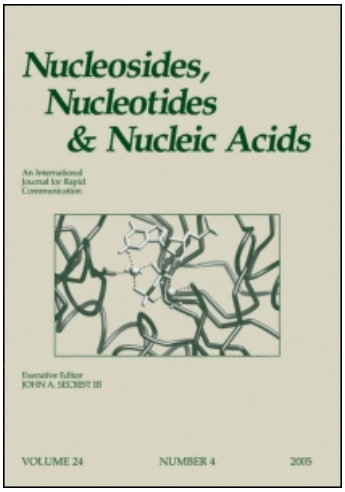

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Michael J. Gait[a]; Jane A. Grasby[b]; Jonathan Karn[a]; Karin Mersmann[a]; Clare E. Pritchard[c]
[a] Laboratory of Molecular Biology, Medical Research Council, Cambridge, UK [b] Krebs Institute, Department of Chemistry, Sheffield University, Sheffield [c] Department of Biochemistry, University of Oxford, Oxford

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SYNTHETIC RIBONUCLEOTIDE ANALOGUES FOR RNA STRUCTURE-FUNCTION STUDIES

Michael J. Gait*, Jane A. Grasby#, Jonathan Karn, Karin Mersmann and Clare E. Pritchard†

Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK

Abstract. The use of synthetic ribonucleotide analogues in structure-function analysis of RNA is reviewed with particular reference to the roles of specific RNA functional groups in hammerhead ribozyme cleavage and in the interactions of the HIV-1 gene regulatory proteins tat and rev with their respective RNA targets TAR and RRE.

Introduction

RNA is highly flexible and can adopt a wide range of three-dimensional structures that allow recognition by proteins and which also facilitate catalytic reactions. It is therefore not surprising that RNA structures play crucial roles in many biological and cellular processes. RNAs fold into stem regions created largely by Watson-Crick base-pairs which are interspersed by numerous bulges and loops. These bulges and loops account for much of the RNA flexibility and help to present RNA functional groups in a variety of contexts suitable for hydrogen-bonding and other polar interactions both externally and internally.

Apart from tRNA, only four RNA structures have been determined to high resolution so far by X-ray crystallography. Three of these have shown the importance of non-Watson Crick base-pairs and other unusual cross-strand

current address: Krebs Institute, Department of Chemistry, Sheffield University, Sheffield, S3 7HF.
† current address: Department of Biochemistry, University of Oxford, Oxford, OX1 3QU.

hydrogen bonds in maintaining RNA duplex structure [1-3]. With the exception of tRNA synthetase-tRNA complexes, very little structural information is yet available for RNA-protein interactions or for catalytic RNA. Therefore, in order to try to assess the roles played by various RNA functional groups in these processes, we have taken a chemical approach. This involves the replacement of single nucleotide residues in the RNA of interest with analogues containing modest functional group changes (deletions, substitutions) such that the hydrogen-bonding character of the nucleotides is altered. By comparing the properties of the modified RNAs with their unmodified partners, information can be derived as to the relative importance of specific RNA functional groups.

The Hammerhead Ribozyme

Hammerheads are independently folded RNA domains that are found in the genomes of several plant viroids and virusoids and which self-cleave during replication[4]. Although the viral cleavage reaction takes place in *cis*, the reaction may be made to occur in *trans* using a suitable arrangement of two or more oligoribonucleotides (Fig. 1a)[5]. Cleavage occurs 3' to the sequence GUB (where B = C, U or A) and requires a divalent metal ion (usually magnesium). The products are oligoribonucleotides containing a 2',3'-cyclic phosphate and a 5'-hydroxyl group respectively. The reaction proceeds with inversion of configuration at phosphorus implying an in line attack of the neighbouring 2'-hydroxyl group and the development of a penta-coordinated transition state [6-9].

The hammerhead includes 13 conserved residues in hammerhead cleavage, the identities of which in most cases cannot be changed without drastic loss of activity [10]. Multiple deoxynucleoside substitutions often resulted in a drastic reduction in k_{cat} whereas K_m was largely unaffected [11,12]. Similar results were obtained upon multiple substitutions with 2'-fluoro-, 2'-amino-, 2'-*O*-methyl- or 2'-*O*-allyl-nucleosides [13-16]. More informative have been single residue substitution studies. For example, deoxynucleoside or 2'-fluoro-2'-deoxy-substitution at G5 or G8 is harmful to catalysis (Fig. 1a) [11,12,15,17], which suggests that the 2'-hydroxyl groups of G5 and G8 may be involved in hydrogen bonding or metal ion chelation [18]. By contrast, replacement of G12 by 2'-deoxyguanosine or by 2'-fluoro-2'-deoxyguanosine enhances the rate of reaction. The effect of deoxynucleoside substitution at A9 is disputed. Whereas Fu and McClaughlin [17] have reported a 2-fold increase in the cleavage upon deoxyadenosine substitution at this position, Perreault *et al.* [12] observed a 20-fold reduction in k_{cat} upon similar modification. Most other positions within the hammerhead conserved residues

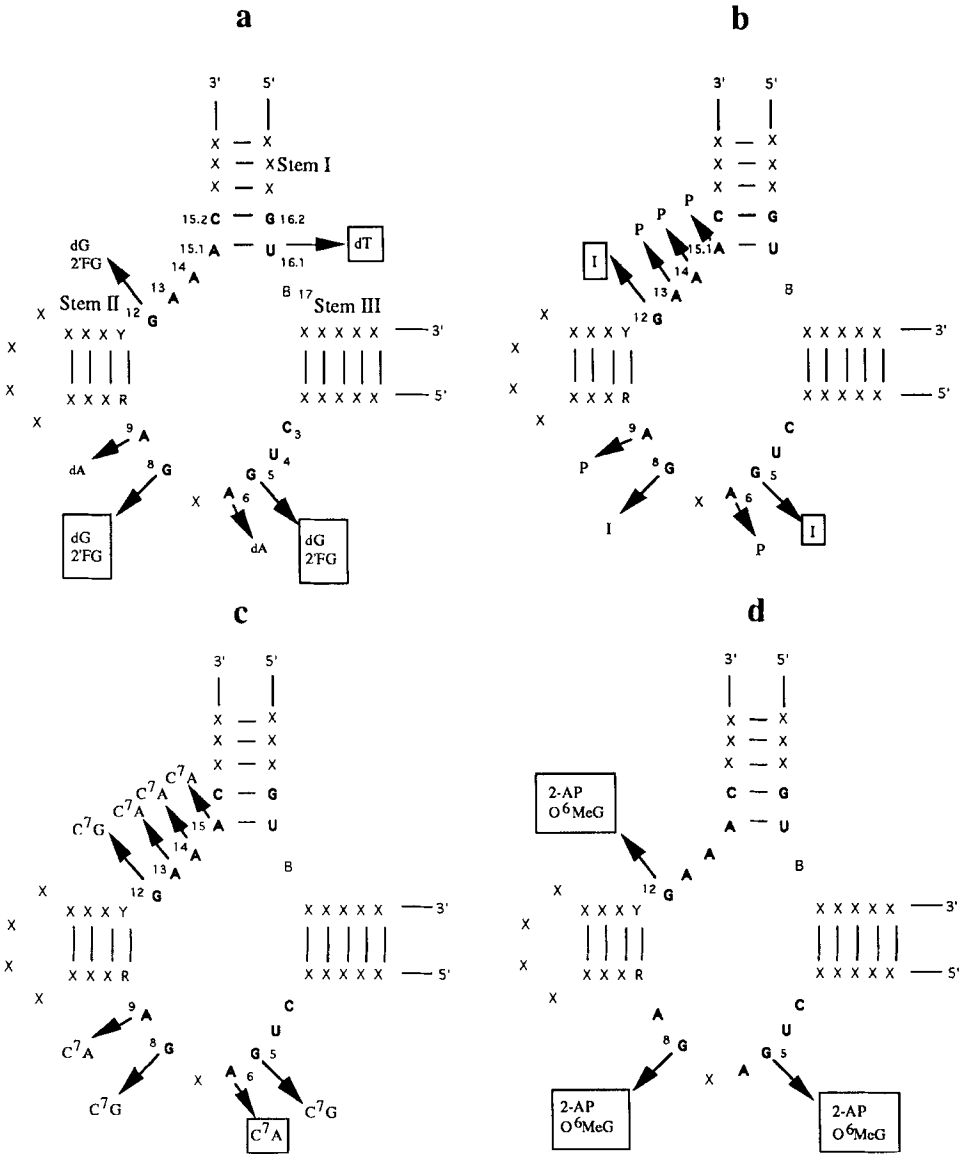

FIG 1. Hammerhead ribozymes in *trans* configuration showing 13 conserved and 3 semi-conserved (B = C, A or U, Y= C or U, R = A or G, X = any base) nucleotides and sites of analogue substitution. Boxed residues indicate those where catalytic efficiency is reduced by at least 100-fold: a) 2'-deoxy- and 2'-fluoro-substitutions, b) purine (P) and inosine (I) substitutions, c) N^7-deaza-A and N^7-deaza-G substitutions and d) 2-aminopurine (AP) and O^6-MeG substitutions (refs. in text).

tolerate 2'-deoxynucleoside or other 2'-modifications rather well and can be substituted, often in unison, with very little loss in catalytic efficiency.

Deoxynucleoside substitutions at the site 5'- of the scissile bond in the substrate completely inactivate the hammerhead [11] as do 2'-*O*-methyl-, 2'-fluoro- and 2'-amino-nucleoside substitutions at this position [9,13]. Deoxynucleoside substitutions within the substrate strand of stems I and III yield reduced rates of reaction due to increases in K_M and to decreases in k_{cat} [19]. The greatest decrease in k_{cat} is produced upon substitution of $U_{16.1}$ by dT which suggests that this residue forms an important hydrogen-bonding contact or has a magnesium-binding function.

The roles of the conserved purine nucleoside residues in the catalytic core have been particularly well studied because these are thought to have the most propensity for cross-strand hydrogen-bonding or for being involved in catalysis. None of the exocyclic amino groups of the conserved adenosine residues (replacement by purine riboside) are essential for catalytic efficiency [17,20,21]. By contrast, removal of an amino group at G_5 or G_{12} (inosine substitution) causes a large drop in catalytic efficiency whilst the effect at G_8 is less marked (Fig. 1b) [20,22,23]. The N^7-positions of guanosine residues appear to be relatively unimportant in catalysis as assessed by N^7-deazaguanosine replacement [21,24] (Fig. 1c). Similarly modest effects of N^7-deazaadenosine substitution have been observed except for a drastic effect at A_6 [25,26]. The importance of the N^1-H and O^6 positions of all three conserved guanosines within the hammerhead has been demonstrated by O^6-methylguanosine and 2-aminopurine substitution (Fig 1d)[23,27]. The results of purine analogue substitition are not consistent with a double mismatch A_9:G_{12}, G_8:A_{13} pairing scheme as was previously proposed[28].

The role of magnesium ion in hammerhead cleavage has been much discussed. A solvated magnesium hydroxide ligand is likely to be responsible for the initial deprotonation of the 2'-hydroxyl group of the residue at the cleavage site [9,29]. Experiments in which the scissile phophodiester bond has been replaced by a phosphorothioate have suggested that the magnesium ion binds to the pro-R_P oxygen of the phosphate in the transition state of the reaction [7-9]. It is possible that a second magnesium ion interacts to stabilize the 5'-oxygen leaving group [7,8,29], a hypothesis which is supported by theoretical calculations [30].

Magnesium ion also undoubtedly plays an important structural role in hammerhead cleavage and some of the identified 2'-hydroxyl groups and functional groups on bases are likely to act as ligands. An increase in K_{Mg2+} upon substitution at a particular site may provide evidence for this. Alternatively if an

increase in magnesium ion concentration can compensate for the reduction in rate observed upon analogue substitution, this suggests that the functional group may bind magnesium ion in the transition state of the reaction. However, views differ over which functional groups are involved in magnesium chelation. One report implicates the hydroxyl groups at G5, A9 and $U16.1$[12], a second points to G8 and G5 but not to A9[17], whereas a third report refutes that any of these are involved[23].

Similarly differing effects of magnesium are reported for hammerheads containing base analogues. Whereas one report suggests that the amino group at G5 may interact with magnesium [17], another reports only small increases in the rates of cleavage as the concentration of magnesium was raised for all inosine- and 2-aminopurine-substituted ribozymes [23]. We observed decreases in affinity for magnesium (increases in K_{Mg2+} values), for hammerheads substituted at G5 or G8 by O^6-methylguanosine but not at G12 (Fig. 1d) [27]. Fu and McClaughlin [26] have suggested that the N^7 of adenosine at A6 is also a site of divalent metal ion binding and have proposed a model for magnesium binding involving various functional groups.

Unfortunately, magnesium titration experiments alone cannot distinguish between whether there is direct or water-mediated magnesium chelation, or alternatively whether an important hydrogen bond is lost which results in repositioning of a magnesium ion chelator. It should also be noted that attempts to relate the effects of functional group modifications on hammerhead cleavage to alterations in binding energies suggest that the predominant effect is on the transition state of the reaction rather than on the ground state, since k_{cat} is usually affected rather than K_M [23,27].

In summary, the extensive analogue substitution studies have pointed to a complex network of hydrogen bonding and metal chelation within the catalytic core of the hammerhead ribozyme. However to date, it has been difficult to reconcile all these results with a satisfying structural model. The results of crystallographic studies on the hammerhead are therefore awaited with particular interest [31,32].

RNA Interactions of HIV Regulatory Proteins Tat and Rev

a) Tat-TAR Interactions

The *trans*-activator protein tat is one of two gene regulatory proteins of the Human Immunodeficiency Virus (HIV) which are essential for viral replication [33,34]. Tat specifically stimulates transcription from the viral long terminal repeat (LTR) and this results in a considerable boost to expression of all HIV proteins.

Tat activity requires an RNA stem-loop known as the *trans*-activation response element (TAR) which is located at the 5'-end of all viral mRNAs. Tat binds to TAR *in vitro* stoichiometrically and with K_d of 1-3 nM. Recognition occurs in the vicinity of a U-rich bulge located near the apex of the stem-loop and mutational analysis has shown that U_{23} in the TAR bulge and the two base-pairs above the bulge (G_{26}:C_{39} and A_{27}:U_{38}) are important to tat binding (Fig. 2a)[35].

Nearly wild-type tat binding can be obtained using a synthetic duplex RNA containing the U-rich bulge and the surrounding base-pairs (Fig. 2b) [36,37]. We have used this duplex as a model for the testing of the importance of particular RNA functional groups to tat binding. For example, replacement of U_{23} by dU or dT had no effect on tat binding, but binding was substantially impaired by substitution by N^3-methyldT [36] or by O^4-methyldT [37] which indicates that the N^1-proton and/or O^4 of U_{23} is important for tat recognition. By contrast, U_{24} and U_{25} seem to act merely as spacers since they may be replaced by propyl linkers without a significant reduction in tat binding [38].

The two base-pairs in TAR immediately above the U-rich bulge are of particular importance for tat recognition. Substitution of G_{26} by N^7-deaza-2'-deoxyguanine or of A_{27} by N^7-deaza-2'-deoxyadenosine substantially reduced binding of tat [37] suggesting that tat recognizes each of these nitrogen atoms located within the major groove of the RNA duplex. These results provide evidence for the hypothesis that the bulged U residues serve to widen the major groove to allow protein insertion and binding [39]. Interestingly, there was no effect of N^6-methyl-A substitution of A_{27} [37]nor of N^4-methyl-C substitution at G26 [40], suggesting that not all potential hydrogen-bonding sites within the major groove at these two base-pairs are recognized by tat.

In the minor groove, the exocyclic amino group of G is the only main distinguishing feature between G:C and A:U pairs. Since there was no effect of inosine substitution at G_{26}, minor groove recognition seems unlikely. Similarly there was no evidence of base contact at either of the two base-pairs below the U-rich bulge as judged by base analogue substitution[37,40].

We have recently used a new method of mapping RNA phosphate contacts to tat and rev which makes use of single methylphosphonate substitutions in model RNAs [41]. In this analogue, a methyl group replaces a non-bridging oxygen atom in an individual phosphodiester linkage. This leads to loss of a potential hydrogen bonding contact as well as the loss of the formal negative charge on the phosphate. The methylphosphonate was introduced into the model TAR RNA duplex by chemical oligoribonucleotide synthesis as a single 2'-deoxy-3'-

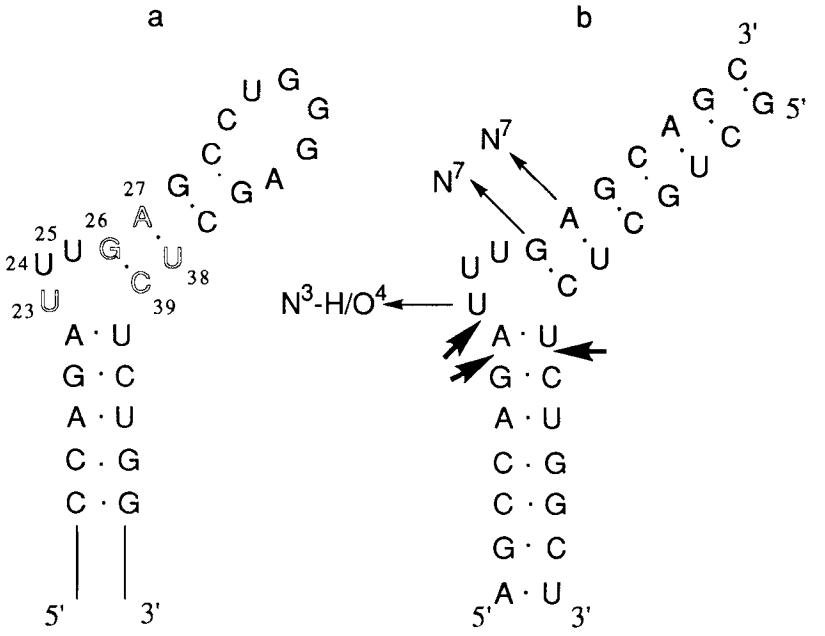

FIG 2. a) Sequence of the apical region of the HIV-1 TAR stem-loop. Outlined residues are those which have been found by mutational analysis to be important to tat binding and to *trans*-activation. b) Model TAR RNA duplex. Thin arrows denote bases where particular functional groups have been identified as crucial to tat binding. Thick arrows denote phosphates where substitution by methylphosphonate (either isomer) is harmful to tat binding.

methylphosphonate nucleoside unit, since a methylphosphonate linkage was not thought to be sufficiently stable when neighbouring a 2'-hydroxyl group. However, it was possible to separate by h.p.l.c. the individual R_P and S_P diastereoisomers such that the effect of each isomer could be tested separately in tat binding. For each methylphosphonate substitution the effect of a control 2'-deoxynucleoside substitution was also tested in tat binding and in all but one case there was an insignificant effect of such substitution.

The results showed that for two phosphates on one strand of TAR (P21 and P22) and for one phosphate on the other (P40) both methylphosphonate diastereomers were inhibitory to tat binding (Fig. 2b)[41]. This is consistent with our proposal that these phosphates may interact with a charged amino acid (such as arginine or lysine which are ubiquitous in the binding regions of the two proteins) or with two different amino acids. The data are generally consistent

with ethylnitrosourea mapping results [35] but because inhibition by steric clashes is unlikely, methylphosphonate substitution experiments give more precise locations for possible phosphate contacts.

A model based on preliminary NMR results for TAR bound by the amino acid derivative argininamide has been proposed to simulate some of the features of tat binding [42,43]. In this model the argininamide inserts into the major groove and forms hydrogen bonds with G_{26} and an unusual base triple is proposed for U_{23}:A_{27}:U_{38}. The chemical substitution studies are mostly consistent with this model but are in better agreement with an alternative NMR-based model obtained in this laboratory which does not include the base triple but instead proposes that these residues are free to interact with tat (G. Varani, personal communication).

b) Rev-RRE Interactions

A second gene regulatory protein of HIV is the regulator of virion expression rev[33]. This protein is responsible for controlling the cytoplasmic expression of partially and unspliced viral mRNAs encoding the viral structural proteins and enzymes. Rev activity is mediated through binding to a cis-acting RNA sequence, the rev-responsive element (RRE), which is present within the *env* gene. The RRE is 351 residues long and forms a complex secondary structure containing several stem-loops. *In vitro* studies have shown that rev binds to the RRE and initially recognizes a duplex region containing a purine-rich "bubble" structure at the base of one of the RRE stems (Fig 3a)[44,45]. Further lower affinity binding to neighbouring, essentially double-stranded RNA regions occurs at higher rev concentrations and both initial high affinity binding and subsequent multimerization along the RRE are required for expression of the rev phenotype[46,47].

Functional groups within the high affinity binding site important to rev recognition have been mapped by chemical substitution experiments using a model RNA duplex (Fig. 3b) [41,46]. Substitution of either G_{104} or G_{128} by N^7-deaza-2'-deoxyguanosine in each case resulted in substantially reduced rev binding, suggesting that, like tat, rev recognizes bases displayed in the major groove of the RNA duplex. However, there was no effect of N^4-methyl-C substitutions in the paired C residues (C_{107} or C_{132}) [40] nor of N^7-deaza-substitutions in G or A residues more distant to the bubble structure[41].

In addition, no evidence has been obtained so far of direct rev recognition of any of the 5 bases in the bulged residues of the bubble. Instead, substitution

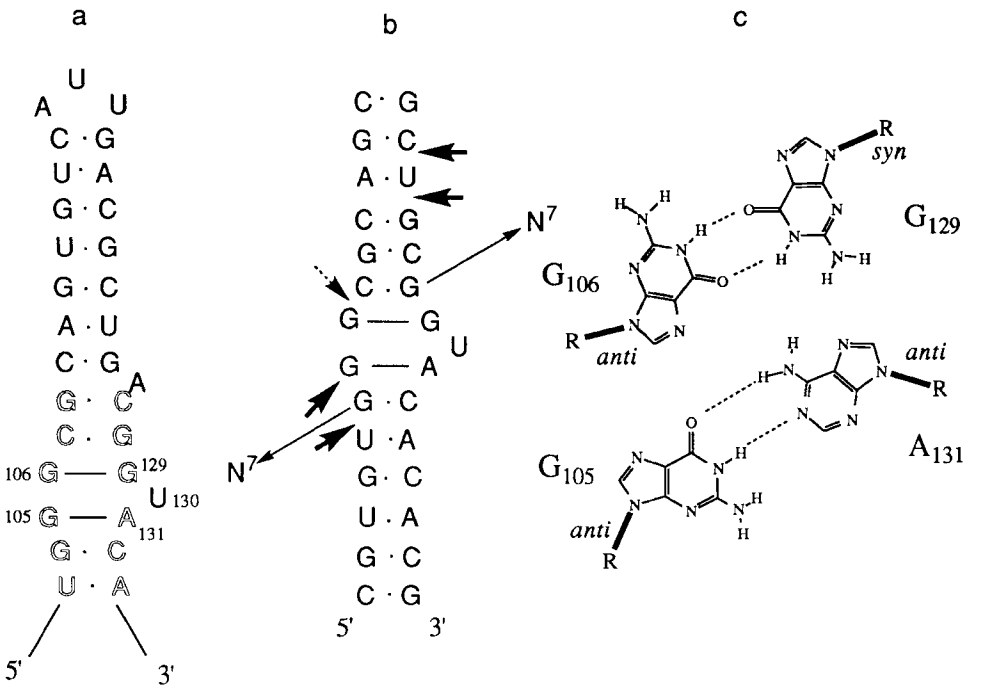

FIG 3. a) High affinity rev binding site of the HIV-1 RRE. Outlined residues are those defined by mutational analysis to be important to rev binding. b) Model RRE RNA duplex. Thin arrows denote bases where particular functional groups have been identified as crucial to rev binding. Thick arrows denote phosphates where substitution by methylphosphonate (either isomer) is harmful to rev binding. Dashed arrow denotes the phosphate where only the R_P methylphosphonate interferes with rev binding. c) Proposed non-Watson-Crick pairing schemes.

experiments with a range of nucleoside analogues (2'-deoxyinosine, N^6-methyl-2'-deoxyadenosine, N^7-deaza-2'-deoxyadenosine, N^7-deaza-2'-deoxyguanosine, O^6-methylguanosine) have provided strong evidence supporting the existence of cross-strand non-Watson-Crick pairs between G105:A131 and G106:G129. The only conformations of these pairs that are consistent with the data are shown in Fig. 3c. By contrast, U130 seems only to be required as a spacer, since it can be replaced by C, N^3-methylU or by a propyl spacer without loss of rev binding [41,46].

Methylphosphonate mapping has also been carried out on the model RRE, in the same way as described for TAR [41]. Both methylphosphonate isomers

showed reduced rev binding when substituted for either of two phosphates on one side of the bubble structure or for either of two phosphates on the other side at sites more distant from the bubble (Fig. 3b). These four sites are likely therefore to be points of interaction with charged amino acids (probably arginines). In one case of methylphosphonate substitution however, P106, only one isomer interfered with rev binding, suggesting that this site hydrogen bonds to an uncharged amino acid or to a main chain amide.

The chemical substitution studies are consistent with recent preliminary NMR studies of similar model RRE duplexes in the presence of rev peptides[48,49]. Both studies support the existence of the particular cross-stand base-pairs as shown in Fig 3c. Further detailed NMR studies of the rev-RRE high affinity interaction are awaited with interest to see whether there is confirmation of our proposals for contact sites in the major groove on the base-pairs flanking the bubble and also on the identified phosphate residues.

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