

Use of synthetic oligoribonucleotides to probe RNA – protein interactions in the MS2 translational operator complex

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ABSTRACT

Synthetic oligoribonucleotides have been used to probe the interaction of MS2 coat protein with the translational operator of the MS2 replicase gene. We have investigated the possible formation of a transient covalent bond between the single-stranded uridine residue, at position –5, and a cysteine side-chain on the coat protein, by the incorporation of a chemically modified residue (5-BrU) at this position. This chemically synthesised operator variant has a binding constant of between 10 and 50 times greater than that of the wild type and is therefore comparable with the tight binding variant having a cytidine substituted at the –5 position. Dissociation kinetics show that the complex with the 5-BrU operator is more stable than the –5C variant; a result which is consistent with the formation of a Michael adduct at the –5 position. In addition, a number of other chemical variants of the operator have been analysed. These include operators incorporating deoxyadenine residues at each of the important single-stranded adenine sites.

Recently the Michael adduct proposal has been challenged on the basis of mutagenesis of the coat protein cysteine residues (1). These results are discussed in the light of our data in support of Michael adduct formation.

INTRODUCTION

Many important cellular events are mediated by the specific interaction of proteins with RNAs. However, despite the importance of this class of interaction we still know very little about the structural details of sequence-specific recognition of RNAs by proteins. Structural studies of RNA-protein complexes have been hampered by 1) the size or complexity of the RNA components, 2) the relative instability of RNAs, and 3) by the unavailability of large quantities of homogeneous complexes. A considerable advance in this field would come from the ready availability of synthetic RNAs and this has led to the development of various RNA transcription systems for the production of RNAs in quantities sufficient for biochemical experiments (2,3,4). However, these systems suffer several disadvantages,

which include the requirement for specific leader sequences for efficient transcription to occur and the restriction to syntheses containing naturally occurring nucleotides. In addition large scale preparations are expensive and the products tend to contain sufficient levels of mis-incorporation as to make structural studies difficult (eg. by broadening NMR line widths).

Clearly an ideal solution would be the direct chemical synthesis of oligoribonucleotides, which would have the potential for the incorporation of novel chemical groupings and cost effective production of material on a large scale. Several routes towards chemical synthesis have been developed. Recently Ogilvie and his colleagues have reported a method for the rapid and efficient construction of ribo-oligonucleotides using automated solid phase methods based on the phosphoramidite chemistry (5) which has proved very successful for the synthesis of DNA oligonucleotides. Since many biochemical laboratories have access to solid-phase oligonucleotide synthesisers this development should allow chemically synthesised oligoribonucleotides to be used in place of *in vitro* transcripts for both functional and structural studies.

We have utilised the Ogilvie method to produce synthetic oligoribonucleotides to study RNA-protein interactions in the MS2(R17) translational repression complex. Uhlenbeck and his colleagues have shown that the coat protein recognition site (translational operator) is limited to a 19 base stem-loop (Fig.1). By the use of sequence variants produced enzymatically it has been shown that essentially all the sequence can be varied without affecting binding. The only requirements appear to be for the secondary structure to be preserved ie. the base-paired stem, and for positions –10 to be a purine, –5 to be a pyrimidine, with adenines at –7 and –4 (6–9).

The pyrimidine requirement at –5 has been explained by the evidence in support of Michael addition to this residue by a protein cysteine during complex formation (10). Changing the –5U to a cytidine results in an RNA with a binding constant approximately 50 times greater than that of the wild type sequence (11). High concentrations of a variety of 5-halogenated pyrimidines and some sulphhydryl reagents inactivate coat protein for RNA binding. The observation of similar nucleoside inactivation in thymidylate synthetase (12) and isoleucine tRNA synthetase (13) has been interpreted as a consequence of the formation of a transient covalent complex between the C-6 of a pyrimidine and a protein cysteine. The tighter binding of the

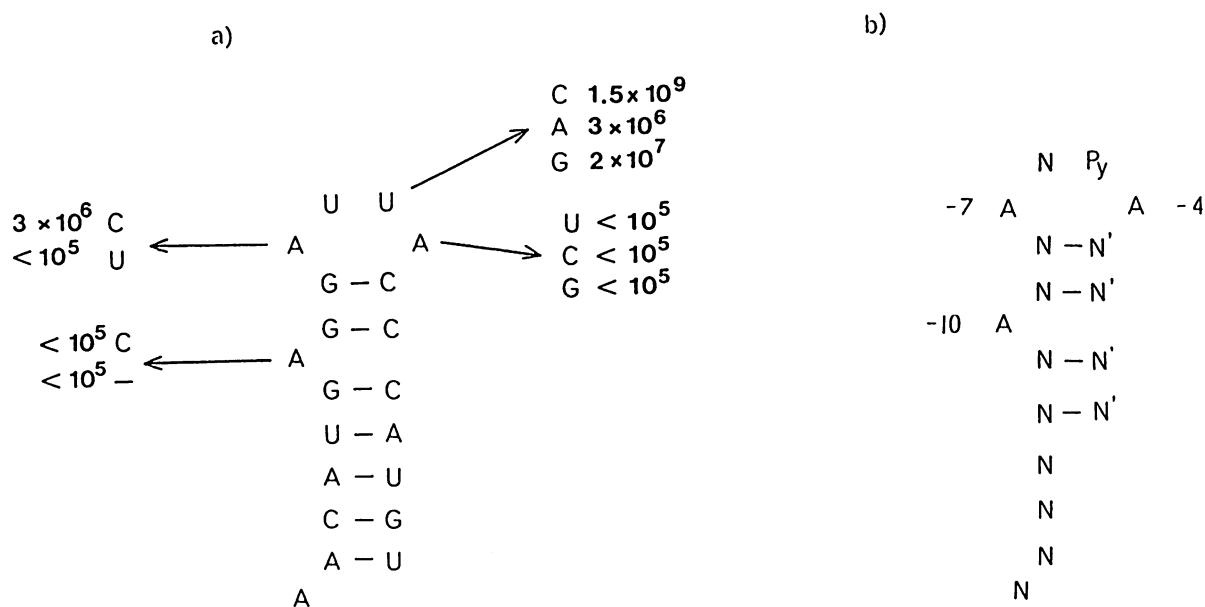


Figure 1 (a) Proposed secondary structure of the stem loop fragment of MS2 RNA which contains all the information necessary to bind coat protein specifically (9). The numbers arrowed on the left hand figure indicate the values of K_a determined for the sequence changes indicated. Wild type $K_a \approx 1 \times 10^8$ M. The sequence, in this figure and throughout the text is numbered relative to the first nucleotide of the replicase gene (+1). (b) Shows the minimum sequence information required for specific binding.

MS2 C-variant is consistent with the fact that the Michael adduct with cytidine is more stable than with uridine (11).

We have been studying the MS2 translational operator complex in order to understand the basis of sequence-specific recognition and binding by the coat protein. To this end we have produced a library of single, double or triple random coding mutations throughout the coat protein gene. The mutant proteins are being analysed for their stability, ability to self-assemble and RNA-binding properties. The determination of the three-dimensional structure of the MS2 capsid by X-ray diffraction techniques should eventually allow all these mutations to be understood in terms of a molecular model of the coat protein. We have also developed a modelling algorithm for RNA stem loop structures (14), which has allowed us to produce a three-dimensional model of the operator fragment. Chemical and enzymatic probes have been used to test key features of the model (*in vitro*) and suggest that it is a good approximation of the structure in solution. The model also explains many of the results of sequence variation experiments in terms of the requirement to maintain the overall operator structure rather than the loss of specific functional groups. The use of synthetic oligoribonucleotides in this system has the advantage that there are excellent *in vitro* assays for the biological function of the RNA fragments (7) and the wealth of knowledge about sequence recognition can be used to direct the synthesis of operators bearing novel functional groups.

MATERIALS AND METHODS

Chemical synthesis of oligoribonucleotides

Chemical synthesis of oligoribonucleotides was performed as described by Ogilvie *et al*(5). All syntheses were performed on a 0.2 μ M scale. Cyanoethyl esters rather than O-Methyl esters were used for the 5-BrU synthesis in order to avoid the use of

Table 1. List of oligoribonucleotides synthesised and the details of each synthesis. Yields are based on trityl assays. All syntheses were carried out on a 0.2 μ mole scale.

Oligonucleotide	Overall Yield(%)	Average Step Yield	Lowest Step	Highest Step	Total (μ g)
1 MS2 wt	40.6	94.8	84.9	100	730
2 MS2 var1 (20mer)	28.5	93.3	83.1	100	473
3 MS2 var2 (-10dA)	34.6	94.0	77.4	100	660
4 MS2 var3 (-7dA)	36.7	94.3	76.5	100	560
5 MS2 var4 (-4dA)	39.2	94.6	80.0	100	700
6 MS2 var5 (-5 5-BrU)	31.8	93.5	70.9	100	560

thiophenol. The DMT-ribonucleotide-phosphoramidites were purchased from Peninsula Laboratories Europe Ltd..

Initial purification of the crude oligoribonucleotides was performed by reverse phase HPLC on a C18 column (Beckman Ultrasphere 5 μ m, 4.6 \times 280 mm). The RNA was loaded onto the column after the final deprotection and desalting step and was eluted with a linear gradient of 7–13% acetonitrile buffered at pH 7.5 with 100mM ammonium acetate. Fractions containing the semi-purified full-length oligoribonucleotide were pooled and then lyophilised.

The synthetic RNA was then labelled either at the 3' terminus with 5' [32 P]pCp and RNA ligase (BRL) (15), or at the 5' terminus with γ [32 P]ATP and T4 polynucleotide kinase (Pharmacia) (16), and purified on 1.5mm thick 20% (w/v) polyacrylamide gels containing 7M urea, 0.1M Tris-Borate pH8.1, 2mM EDTA (17).

Synthesis and protection of 5-bromouridine

5-BrU was synthesised as described by Levene *et al* (18), 5' and 2' protection was as described by Hakimelahi *et al* (19), and 3' protection as described by Ogilvie *et al* (5).

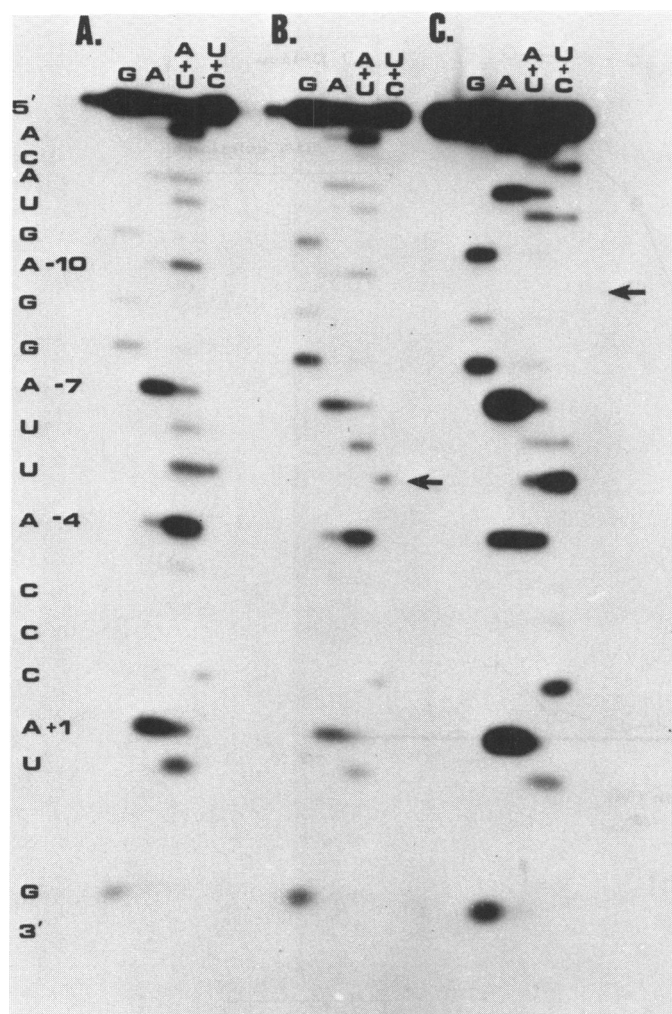


Figure 2 Enzymatic sequencing of synthetic oligoribonucleotides. Gel purified RNAs radiolabelled at either the 5' or 3' ends were sequenced using the sequence specific nuclease technique (21). Panel A: MS2 wt, Panel B: -5 (5-Br) uridine variant and Panel C: -10 deoxy A variant. Arrows indicate positions of absent or aberrant bands due to the presence of the modified nucleotides.

Base compositional analysis

As described by Eadie *et al* (20).

Binding assays

Filter binding affinity and dissociation assays were performed as described by Carey and Uhlenbeck (7).

Gel retardation

Complexes were formed between $\approx 10\text{pM}$ RNA (5' labelled) and varying concentrations of coat protein in $25\mu\text{l}$ 50mM Tris-acetate pH 7.6, 1mM magnesium acetate, 10% (w/v) ficoll, 0.05% (w/v) xylene cyanol and bromophenol blue, for 3hrs at 2°C . The samples were then run on 10% (w/v) acrylamide gels containing 50mM Tris-acetate pH 7.6, 1mM magnesium acetate for 3hrs at 4°C with re-circulation of the running buffer. Gels were pre-run for 30min prior to loading of the samples. All gels were dried under vacuum before autoradiography.

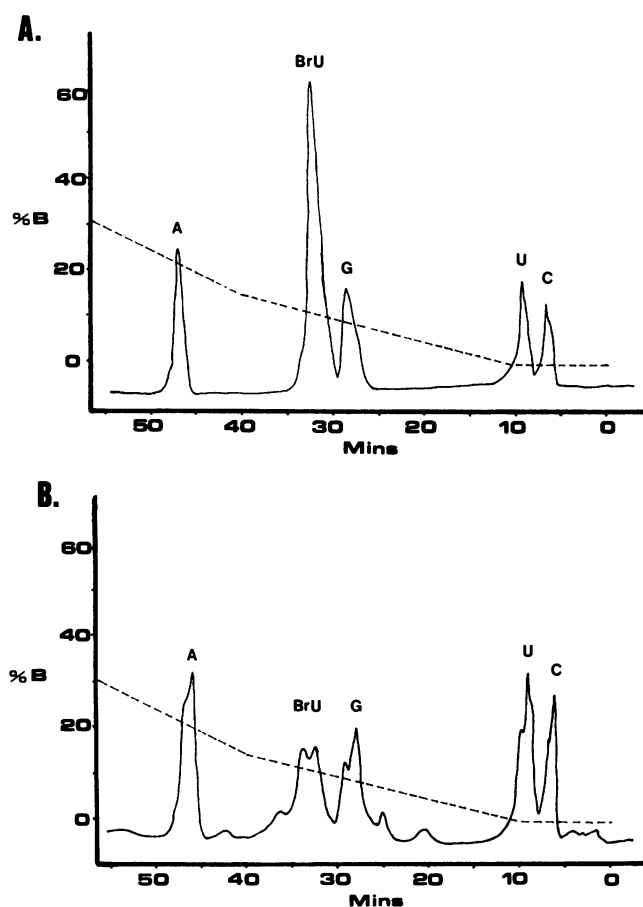
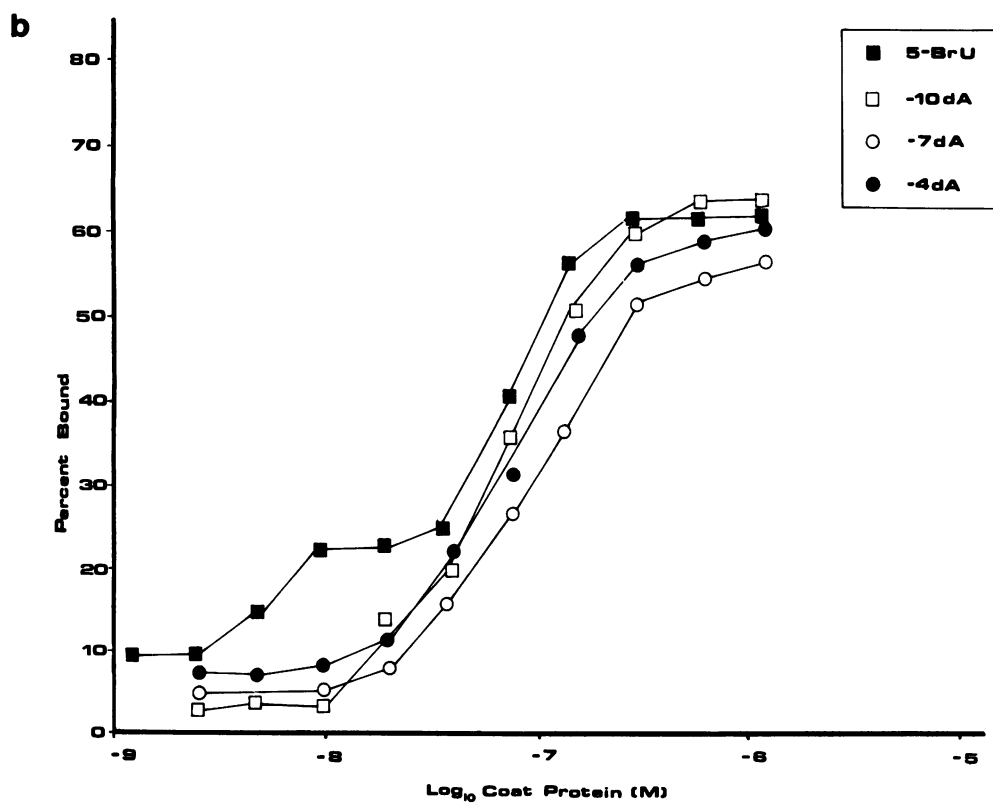
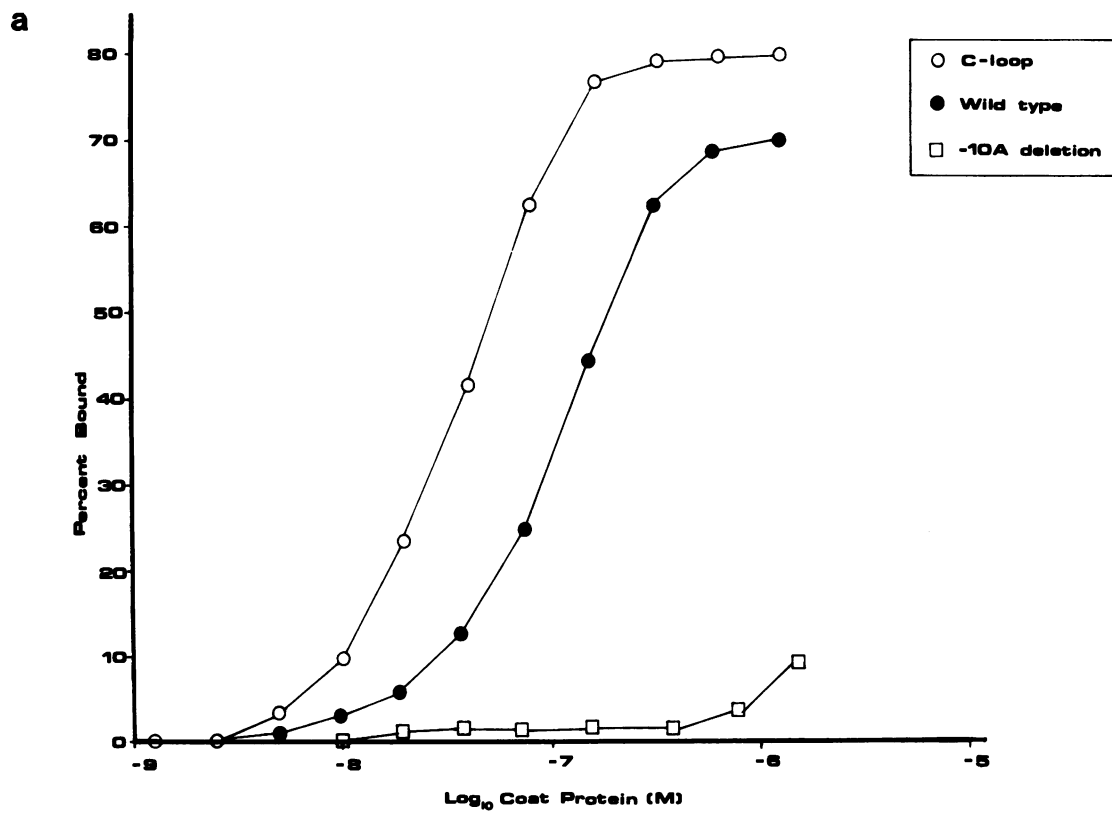


Figure 3 Base composition analysis of synthetic oligoribonucleotides. HPLC profiles of a) ribonucleoside standards and b) variant 5 (-5, 5-BrU) digested and processed as described in Materials and Methods. Nucleosides were fractionated on a C18 reverse phase column (250×2.5 mm) with a gradient of 0 to 90% methanol in 50mM Na_2HPO_4 pH5.5. Gradient profile (dotted line) was 0% B, 10 min: to 15% B, 30 min: 15% B for 10 min: to 45% B over 30 min: then to 100% B over 30 min. Profiles are absorbance traces at 254nm. Peak fractions were pooled, lyophilised and analysed by thin layer chromatography alongside standards. The doubling of the peaks in trace B was due to a problem with the C18 column, TLC of both sections of each peak showed that they contained identical material.

RESULTS AND DISCUSSION

Chemical synthesis of oligoribonucleotides

All RNAs used in this study were synthesised chemically using the methods described by Ogilvie *et al* (5), with the modifications described in Materials and Methods (Table 1). Initially the final desalting step after treatment with TBAF in THF was also omitted and instead, the crude deprotected RNA was applied directly onto a C18 reverse phase HPLC column and eluted with a linear gradient of 7-13% acetonitrile buffered at pH 7.5 with 0.1M ammonium acetate. However, this led to aggregation problems between the RNA fragment and the column matrix due to the presence of tetrabutyl ammonium ions. To prevent this the deprotected material was passed through Dowex 50 (NH_4^+ form) before application to the C18 column. This was much faster than desalting by gel filtration on Sephadex G-10. The HPLC step had the benefit of partially purifying the full length RNA from failure sequences and non-deprotected RNA, prior to end-labelling and final purification by gel electrophoresis (17).



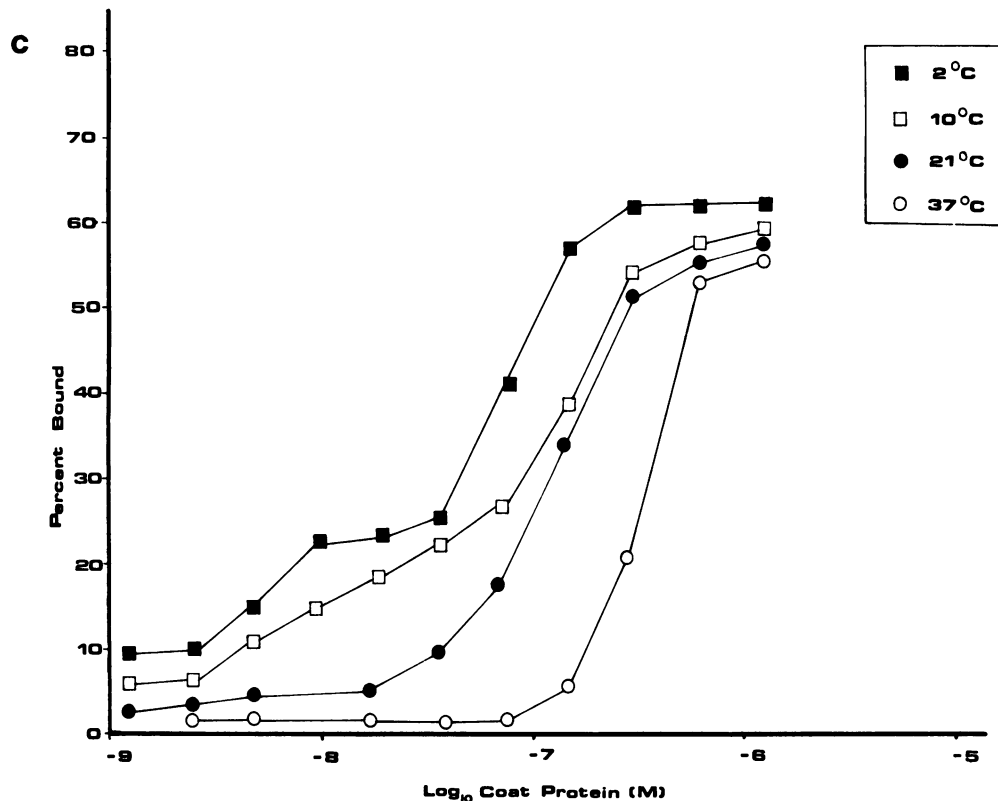


Figure 4 (a) Coat protein excess binding curves for sequence variants of the MS2 operator. Filter binding assays were performed as described (7). (b) Affinity curves of the synthetic operators containing variant nucleotide or sugar residues. (c) The effect of temperature on the affinity of coat protein for the 5-BrU variant.

The average step-wise yields as determined by trityl release were 90–98% giving overall yields from 20–48% (Table One). There was considerable variability between different batches of phosphoramidites. The guanosine reagent was particularly troublesome. Solutions of this compound often precipitated on standing but after subsequent filtration gave high coupling efficiencies. Similar problems were encountered in the initial commercial supplies of the deoxy series of phosphoramidites and have been shown to be due to excess phosphitylating reagent (A. Andrus, personal communication). Synthesiser set-up chemicals from different suppliers also gave variable yields. The best results to date have been obtained with chemicals from Applied Biosystems. The results with the 5-bromouridine compound demonstrated the potential of this chemistry for utilising non-standard nucleotide groupings. The step yields for this compound were noticeably lower than those for the commercial reagents. This was probably due to the impurity of the brominated compound, since subsequent repurification resulted in higher coupling efficiencies. This compound is unstable and decays with time. Our results suggest that phosphoramidite purity is the major factor in coupling yields. The results with the deoxyadenosine derivatives suggest that all the chemical variants already available in the deoxy series could be incorporated into RNA fragments by this method.

Sequence and base composition analysis

All RNAs were sequenced enzymatically (21), and terminal nucleotides confirmed by P1 (5' terminus) and T2 (3' terminus) digestions followed by thin layer chromatography. Fig. 2 shows

Table 2. Thermodynamic and kinetic properties of coat protein binding to wild type and variant operator fragments.

Operator	k_{ass} M^{-1}	k_{diss} min^{-1}	$t_{1/2}$ min^{-1}
Wild type	10^7	0.15	0.70
-5 C variant	6×10^7	1.7×10^{-3}	408
-10dA	10^7	—	—
-7dA	10^7	—	—
-4dA	10^7	—	—
-5-5BrU	2×10^8 & 2×10^7	1.6×10^{-3}	433
-10A	deletion no detectable affinity		

enzymatic sequencing gels of the oligoribonucleotides used. All the synthetic fragments are correctly recognised at the positions of the naturally occurring bases. The 5-BrU residue is cleaved only by the *B. cereus* enzyme (normally specific for C & U) and the deoxyadenine positions are not cleaved. In addition to sequence analysis, the oligoribonucleotides were digested to completion with snake venom phosphodiesterase and alkaline phosphatase, and the resulting nucleoside mixture separated by reverse phase chromatography. The nucleosides were eluted with a gradient of 0–90% methanol buffered at pH 7.0 with 50mM phosphate buffer. Fig. 3 shows a representative HPLC trace of such a base composition analysis for the 5-BrU containing oligoribonucleotide.

Each peak was assigned by comparison with elution times of nucleoside standards, and by direct comparison on thin layer chromatography plates (data not shown).

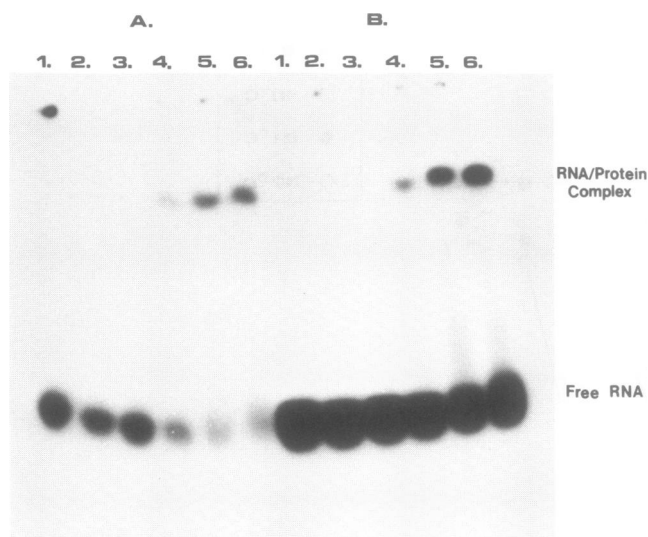


Figure 5 Analysis of complexes between coat protein and A) C-loop; B) 5-BrU 19 mer RNA by polyacrylamide gel electrophoresis. Coat protein concentrations are lane 1; zero, 2; 1nM, 3; 10nM, 4; 100nM, 5; 1 μ M, 6; 10 μ M. RNA concentrations were kept constant at \approx 10pM. The faster moving band on the autoradiogram corresponds to free 19 mer and the slower one to the RNA-protein complex.

Kinetic and Thermodynamic Analysis

Carey and Uhlenbeck (7) have established a filter binding assay to study the interaction of coat protein with operator fragments. Analysis of a synthetic wild-type sequence, the $-5C$ variant, and the -10 variant (Table 1), by these methods, showed that the synthetic oligoribonucleotides were recognised normally by coat protein. That is the half-maximal binding and saturation of both the wild-type and tight-binding $-5C$ variant affinity curves were approximately equal to that seen for operator fragments derived from the phage (6) or from *in vitro* transcription assays (9) and is a reflection of the intrinsic RNA-protein affinity and the retention efficiency of the filter (Fig. 4a). The -10 variant did not bind coat protein at concentrations where binding is specific. Substitution of adenines at -4 , -7 and -10 by dA resulted in small decreases in the affinity for coat protein suggesting that the operator structure is not greatly disturbed by these changes (Fig. 4b) and that there are no direct contacts made between the protein and the 2' hydroxyl group.

The 5 BrU derivative shows a complex affinity curve with an apparent initial saturation of some 20% of the input RNA with a half-maximal concentration of coat protein of 10^{-8} M followed by a second increase in binding with an apparent affinity slightly lower than wild-type but with an identical saturation. Affinity measurements at different temperatures (Fig. 4c) showed that as the temperature is increased the complex behaviour disappears to produce an apparently single binding curve. As with the wild-type sequence the apparent affinity decreases with increasing temperature. At temperatures below 21°C there appears to be two classes of operator fragment as judged by their affinity for coat protein. The amount of the high affinity component is apparently reduced by increasing temperature.

The kinetics of the interaction between coat protein and the RNA variants have been assessed by determination of the rates of dissociation of the complexes (Table Two). The rate of

dissociation of the complex with the 5 BrU derivative reveals a dramatic increase in the half-life as predicted as a consequence of Michael adduct formation with a nucleotide containing the electron withdrawing substituent at the 5 position.

The kinetics of operator-coat protein dissociation were reflected by the behaviour of the complexes in polyacrylamide gels (Fig. 5). The C-variant complex is sufficiently stable that it can be identified as a distinct retarded band. The 5 BrU derivative produced a similar retarded species, which co-electrophoresed with the C-variant complex. No other species were detected in the gel which might have accounted for the high and low affinity interactions between this RNA variant and the coat protein.

CONCLUSIONS

Several approaches toward chemical synthesis of ribo-oligonucleotides have been reported in the literature (5, 22, 23, 24, 25). However, most of these have been restricted by their complexity to synthetic organic chemistry laboratories and are not readily available to biochemists. The approach of Ogilvie (5) has the advantage that the synthesis uses phosphoramidite chemistry similar to that used for the solid phase synthesis of DNA oligonucleotides. This allows ribo-oligonucleotides to be synthesised automatically using existing solid-phase DNA synthesizers which are widely available.

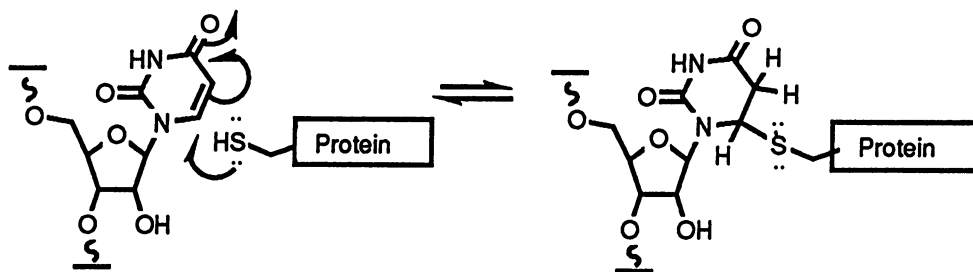
Various problems occur with the synthesis and deprotection of ribo-oligonucleotides which are not relevant to the deoxy series. The most important of these is interconversion of phosphate esters between the 2' and 3' positions. The use of 2' silyl protected nucleotides, which are subsequently deprotected in the presence of fluoride ions appears to have overcome this problem (5). It is, however, important to test the synthetic products of this methodology extensively before its use becomes routine. The synthesis of a tRNA(70mer) which is correctly charged with its cognate amino acid has been reported (26), together with the large scale (20 μ mole) synthesis and purification of a self-complementary 12mer (23). This duplex has been subjected to high-resolution 2D 1H NMR which failed to detect significant chemical inhomogeneity.

Here we report the use of this synthesis technology to probe the details of a well-characterised RNA-protein recognition event in the formation of the MS2 translational repression complex. This complex has the advantage that it has been extensively studied in terms of sequence specificity (9) and allows detailed comparisons to be made with the synthetic products.

Our results of filter-binding with sequence variants suggested that the synthetic ribo-oligonucleotides are correctly recognised by the MS2 coat protein with K_a 's identical, within experimental error, to those obtained with the operator RNA transcribed *in vitro*. Furthermore, saturation values for coat protein binding indicate that the bulk of the material is competent for binding. The synthetic ribo-oligonucleotides are also correctly recognised by the sequence-specific RNases used to confirm their sequences. These reactions appear highly specific since fragments containing chemically variant groups (5 BrU or deoxy adenine) are mis-recognised or resistant to cleavage.

The versatility of this approach for analysing RNA-protein interactions has been demonstrated by the incorporation of chemically modified groups. The filter binding data with the 5 BrU derivative strongly suggest that a Michael adduct forms between the coat protein and the operator fragment. This

a.



b.

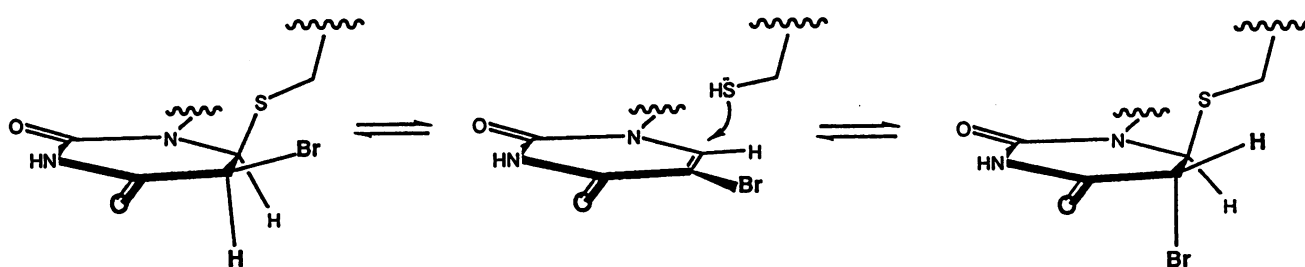
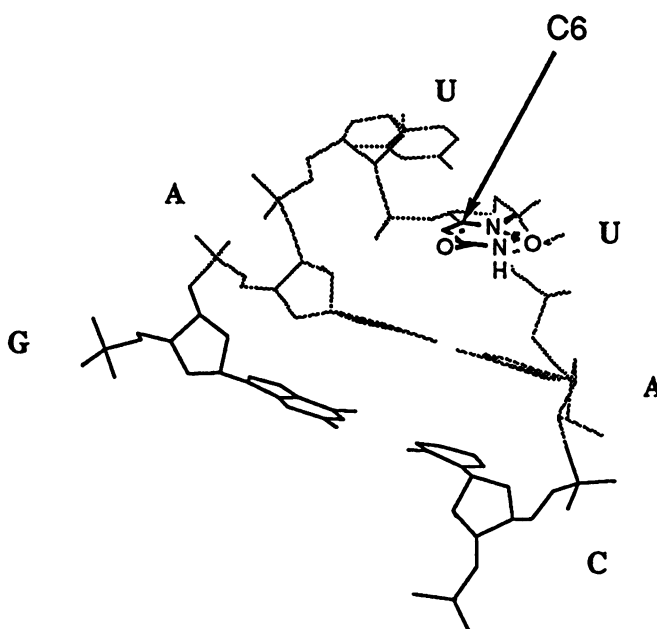


Figure 6 (a) Proposed mechanism for formation of Michael adduct between protein cysteine and the -5 pyrimidine. In the light of results from protein mutagenesis experiments (1), the Michael donor need not necessarily be cysteine. (b) Framework representation of the structure of the MS2 operator fragment deduced from molecular modelling and structure probing experiments (14). The position of the C6 atom in the model structure is indicated. Below is a diagram of the possible diastereomers which may form with the 5-bromouridine derivative. Formation of the cis diastereomers might be expected to be irreversible.

suggestion has recently been challenged by Peabody (1) on the basis of coat protein mutagenesis experiments. He reports that *in vivo* binding to the operator fragment still occurs when both coat protein cysteine residues (positions 46 and 101) are mutated to groups incapable of Michael addition. (Chemical modification/inactivation experiments strongly implicated a cysteine residue as the potential Michael donor (27)). Our results are consistent with the formation of such a Michael adduct at the -5 position, but do not yield any information about the Michael donor. The absence of adduct formation may not completely abolish coat protein binding since Uhlenbeck has shown that substitution of either purine at position -5 results in a fragment with ten to twenty fold lower affinity, which might be overcome at high protein concentrations. We have constructed mutants at both Cys⁴⁶ and Cys¹⁰¹ and are currently examining their interaction with both wild-type and 5 BrU substituted operator fragments (Macedo and Stockley, in progress).

The behaviour of the 5 BrU derivative in the filter-binding assay apparently reveals the presence of two-components in the reaction mixture. This effect is temperature-dependent, and quite reproducible. Several possibilities which might account for this result have been considered. These are i) bromine substitution leading to a tautomeric shift of the base at the -5 position, ii) differential exposure of the target C6 atom due to hindered rotation of the substituted base around C1', iii) preferential interaction by the coat protein with one of the two possible diastereomers which could be formed during Michael addition (Fig.6) and iv) two differing conformations of the coat protein ligand. We exclude, i) because the NMR spectrum of the protected 5-bromouridine phosphoramidite is consistent with the presence of only the keto form, and iv) because if the effect were due to the presence of several protein conformations it should have been observable with the other RNA fragments. A molecular model of the operator fragment has been built which suggests that the C6 atom is not accessible from the solvent (14) consistent with ii). In order to discriminate between the hindered rotamer and the diastereomer effects we are synthesizing other Michael acceptors, e.g. 5-CN.U, which retain the electron withdrawal properties of Br but are much smaller (Goodman et al, in progress).

The results with the deoxyadenosine substitutions show the advantage of compatible chemistry between the deoxy and the ribo phosphoramidite series. In particular for RNA's, it should be possible to examine the role of the 2' hydroxyl group by synthesizing mixed ribo-deoxy oligonucleotides. This might be especially useful in examining RNA's in which the 2' hydroxyl group is suspected of an important structural or functional role (such as formation of a H-bond with a protein or other ligand, or direct participation in a chemical reaction). Deoxy substitution at all three important adenine positions in the MS2 translational operator suggests little or no effect on this recognition event.

These data confirm the usefulness of the Ogilvie approach to chemical synthesis of ribo-oligonucleotides and highlight the potential for its application to a wide number of exciting problems involving RNA.

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