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A comparison of two phage coat protein–RNA interactions

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ABSTRACT

The interaction between the coat protein of the group I bacteriophage fr with its translational operator site is compared with the previously studied R17 interaction. The sequence of the two RNA binding sites differ by 2 of 20 nucleotides and two coat proteins by 17 of 129 amino acids. An analysis of the binding of fr coat protein to 24 operator variants revealed that the two proteins recognize operator sequences in virtually the same way. However, fr coat protein binds to nearly every RNA 6 to 14-fold tighter than R17 coat protein. Since the fr operator is a weaker binding variant and the fr coat protein shows a different temperature dependence of binding, it is unlikely that the two systems have different K_as *in vivo*. RNA fragments containing the operator sequences can initiate the capsid assembly with both fr and R17 coat protein. Surprisingly, the two coat proteins can form a mixed capsid *in vitro*.

INTRODUCTION

The RNA bacteriophage fr is of the same antigenic class (group I) as its better characterized relatives, R17 and MS2. The available sequence of the fr genome indicates that it has diverged substantially from the very similar R17 and MS2 sequences (1). Not only are various insertions and deletions observed in the non-coding sequences, but 20% of the nucleotides in the coat protein gene differ between MS2 and fr. Although many of these changes are silent, the 17 amino acid substitutions between R17 and fr result in proteins with very different electrophoretic mobilities.

In a manner analogous to R17, translation of the fr replicase gene is negatively regulated late in phage infection by the binding of fr coat protein. The site of fr coat protein binding to the RNA (translational operator) has been identified to be a hairpin containing the replicase initiation codon (1). The sequence of this fr hairpin differs from corresponding R17 sequence by two single stranded nucleotides. MS2 coat protein, which has the same sequence as R17 coat protein, has been shown to bind the fr operator (1).

Our laboratory has extensively studied the R17 coat protein-translational operator interaction as a model of a sequence specific RNA-protein interaction (2,3). Here the corresponding fr coat protein-RNA interaction is examined to see whether the 17 amino acid changes in the coat protein and two nucleotide changes in the operator alter the general properties of the interaction.

MATERIALS AND METHODS

Phage and Coat Protein

fr phage was obtained from Dr. E. Gren. The fr and R17 phages were prepared (4), and the coat proteins were isolated from the respective phage (2). The purity of two phages were confirmed by agarose gel electrophoresis (5) and the coat proteins by SDS gel electrophoresis. To prepare oxidized fr and R17 coat proteins, 20 μM coat protein was incubated with 25 mM H_2O_2 in TMK buffer (0.1 M Tris-HCl, pH 8.5 at 2°C, 10 mM $Mg(OAc)_2$, 80 mM KCl) at room temperature for 5 hours and 4°C for 16 hours.

RNA Fragments

Internally ^{32}P-labeled fragments 2 and 2-N were synthesized by the T4 RNA ligase method (3,6). Fragments 1 and 3-9 were synthesized by transcription of synthetic DNA templates by T7 RNA polymerase (7). Fragment 10 was synthesized in a 10 ul reaction containing 1 ug EcoRI digested pGU6 (8), 700 uM rNTP, (12.5 Ci/mmole α-^{32}P CTP), 40 mM Tris-HCl, pH 8.1, 6 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.1 mg/ml BSA and 60 units/ul T7 RNA polymerase. The reaction was incubated at 37°C for 1 hour. Fragments 10-1 and 10-2 which differ from fragment 10 by having a different number of phosphate groups at the 5' terminus and fragment 10-4 which has an extra 5' terminal A residue were synthesized by including 5 mM GDP, GMP, or ApG to the reaction mixtures. Fragment 10-3 was obtained by removing the terminal triphosphate groups of fragment 10 by bacterial alkaline phosphatase (7). Fragment 11 is a 109 nucleotide RNA containing the sequence of 10 at its 5' end and was synthesized in the same manner as fragment 10 except that pGU6 DNA was cleaved by PvuII. All the RNA fragments were purified on a polyacrylamide urea gel. Fragment 11 was further purified by passing it over a Sephadex G-50 column, equilibrated with 1 mM EDTA, 0.3 M NaOAc, 10 mM Tris-HCl, pH 8.0 at 25°C, and then precipitated with ethanol. Each fragment was heated at 85°C for 1 minute and cooled to room temperature for five minutes before use.

Filter Binding Assay

The equilibrium association constants between coat protein and RNA fragments were determined by the nitrocellulose filter binding assay (7,9).

Most assays were carried out in TMK buffer (0.1 M Tris-HCl pH 8.5, 10 mM $Mg(OAc)_2$, 80 mM KCl and 80 ug/ml bovine serum albumin) at 2°C. Since the fr coat protein is able to form capsid in the absence of RNA at concentrations greater than 3.5 uM under the conditions of this assay (10), the fr coat protein concentration in the assay ranged from 5 pM to 500 nM. Since under the assay conditions both the R17 and the fr coat proteins are in the dimer form and each dimer binds one RNA (8), coat protein concentration is expressed in moles of coat protein dimer. Duplicate assays using the same set of coat protein dilutions gave K_a values within a factor 2. The precision in K_a for independent coat protein dilutions was a factor 3. Each K_a reported is the mean value of three to ten independent determinations.

Capsid Formation Reaction and Gel Assay

10 uM coat protein was mixed with 0.13 uM of fragment 11 and incubated in TMK buffer at 4°C. After 12 to 16 hours, 20 ul of the reaction mixture was mixed with 4 ul of the loading dye (30% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue) and applied to 1.2% agarose gel (14x14x0.8 cm). Electrophoresis was carried out in 50 mM potassium phosphate pH 7.0 and 1 mM $MgCl_2$ at 120 to 150 mA for 6 to 10 hours (5). The capsid was visualized by coomassie blue staining.

RESULTS

RNA Structural Requirements for fr Coat Protein Binding

Based upon the data of Cielens *et al.* (1), the RNA hairpin loop corresponding to the -23 to +4 positions of the fr replicase gene should be sufficient to bind fr coat protein. Fragment 1 (Fig. 1) corresponds to -17 to +4 of the fr operator with an additional GpG at the 5' terminus in order to improve transcription by T7 RNA polymerase (11). As shown in Fig. 2A, fr coat protein binds fragment 1 with a $K_a = 1.9 \times 10^9 M^{-1}$ which is similar to what is observed in the R17 system and suggests that fragment 1 contains the entire binding site. Fig. 2A also shows that in agreement with Cielens *et al.* (1), fr coat protein also binds the R17 operator (fragment 2) and, interestingly, that the K_a is actually somewhat tighter at 7.2×10^9 M^{-1}. This preference of fragment 2 over fragment 1 is also observed for R17 coat protein (2), but as shown in Fig. 2B, both fragments bind ten fold weaker to this protein.

In order to examine the RNA structural requirements for fr coat protein binding in greater detail, a collection of available R17 operator variants (2,3,7) were assayed with fr coat protein. The resulting K_a values were

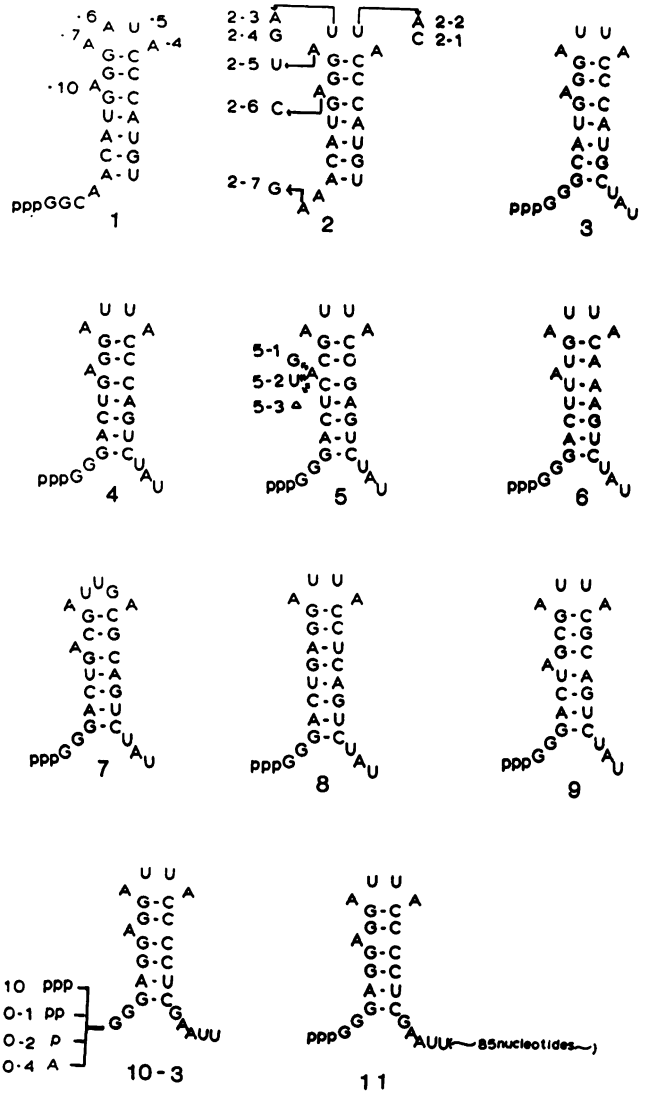

Figure 1: Sequences and possible secondary structures of RNA fragments including the fr operator (fragment 1) and the R17 operator (fragment 2). The numbering system in 1 is used throughout.

compared in Table 1 with K_a's redetermined with R17 coat protein. It is clear that fr coat protein interacts with RNA in a very similar way as R17 coat protein. This is best shown by the fact that the ratio of the K_a's of the two proteins to virtually every fragment is between 4 and 14. Several individual results require comment.

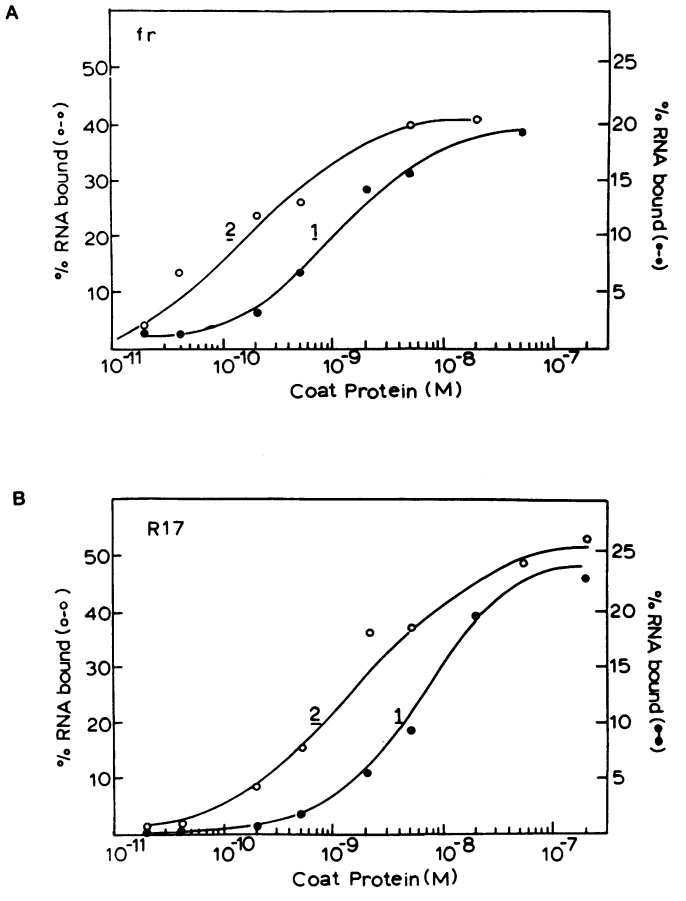

Figure 2: Protein excess binding curves at 2°C in TMK buffer. A: fr coat protein to fragment 1 (•), fragment 2 (o); B: R17 coat protein to fragment 1 (•), fragment 2 (o).

The fact that position -17 is a C in the fr operator and an A in the R17 operator is not relevant for binding to either coat protein. When C_{-17} in fragment 1 is substituted to A_{-17} in fragment 2-3, the K_a does not change. In addition, changing A_{-17} to a G doesn't alter K_a (compare fragment 2 with 2-7). In fact, it does not appear that position -17 is even part of the operator since fragment 10-3, which is missing position -17, has virtually the same K_a as fragment 10-4 which has an A_{-17}. Fragments 10, 10-1 and 10-2 which are missing A_{-17} and have a 5' terminal tri-, di- and monophosphate respectively also have similar K_as. These findings do not agree with our previous data (2)

Table 1. K_a of fr and R17 Coat Proteins Binding to RNA Fragments.

Fragment	K_a (nM-1) fr coat protein	K_a (nM-1) R17 coat protein	Relative K_a fr/R17
1	1.9	0.14	13.6
2	7.2	0.6	12
2-1	520*	72*	7.2
2-2	0.052	0.006	8.7
2-3	2.8	0.2	14
2-4	2.0	0.2	10
2-5	-†	-	-
2-6	-	-	-
2-7	7.2	0.6	12
3	7.6	0.84	9
4	7.6	0.92	8.3
5	3.8	0.39	11.2
5-1	1.3	0.34	3.8
5-2	-	-	-
5-3	-	-	-
6	0.8	0.084	9.5
7	-	-	-
8	-	-	-
9	-	-	-
10	1.3	0.24	5.4
10-1	1.3	0.34	3.8
10-2	1.3	0.22	5.9
10-3	2.2	0.28	7.9
10-4	1.5	0.24	6.3

† signifies no detectable binding ($K_a < 10^5$).
* data estimated from kon/koff

in which the K_a to R17 coat protein decreased more than 1000-fold when the -17 residue was deleted. Since the data here is based on a larger number of molecules made by the more reliable T7 transcription method, we believe that the previous result (2) is incorrect.

The situation at position -6 is interesting. The fr operator has an A this position while R17 has a U. As already shown in Fig. 2, the U_{-6} operator binds to both proteins three-fold more tightly than the A_{-6} operator. Substituting the A (fragment 2-3) with a G (fragment 2-4) has no effect on K_a. Thus the substitution pattern is identical in the two systems, but in contrast to R17 the fr operator sequence does not contain the tightest possible nucleotide at this position.

The substitution pattern at position -5 is also the same for both proteins. Changing U_{-5} to an A (fragment 2-2) reduces K_a 100 fold while changing it to a C (fragment 2-1) increases K_a 70 fold. A transient covalent

bond between coat protein and RNA has been hypothesized for this position (12,13). The $K_a = 5 \times 10^{11}$ for the binding of fr coat protein to 2-1 is the tightest RNA-protein interaction measured to date. Neither phage use this tight contact in their operator sequence.

The only position where R17 and fr coat proteins appear to interact with RNA somewhat differently is at the bulged A_{-10}. This nucleotide is essential for protein binding since K_a decreases more than 1000-fold when A_{-10} is either deleted (fragment 5-3), base paired with a U residue (fragment 8), substituted by a pyrimidine residue (fragments 2-6 and 5-2) or moved to another position in the helix (fragment 9). However the replacement of A_{-10} of fragment 5 by a G residue (fragment 5-1) results in a three-fold decrease in K_a to fr coat protein. In contrast to this result, R17 coat protein binds the two fragments equally well (Table 1). This ability of fr coat protein to better distinguish a G from an A indicates a slightly different interaction between the protein and the RNA in this portion of the operator.

Properties of the fr coat protein - RNA Interaction

Since the two coat protein-operator interactions perform identical functions *in vivo*, it is somewhat surprising that they show a three fold difference in K_a *in vitro*. The possibility that the fr system might have different solution properties prompted a study of the pH, salt and temperature dependence of K_a for this interaction. The K_a between fr coat protein and fragment 2 was determined at different pH values and the optimal pH for the interaction was found to be very similar to R17 (9) with an optimum between pH 8.0 and 9.0.

The salt dependence of the K_a for the coat protein-fragment 3 interaction was measured by varying the KCl concentration. The log K_a versus log M^+, where M^+ is the sum of the concentrations of KCl and Tris-HCl, is plotted for both systems in Figure 3 and the lines were drawn according to the least-squares fit of data between 0.23 and 1.08 M M^+. The slope of the fr data is 3.0 which is quite similar to the value 2.8 for the R17 data. While interpretation of these values in terms of the number of ionic contacts between coat protein and RNA requires additional assumptions (14), it is clear that this number is virtually the same for the fr and R17 systems. Thus, the tighter binding in the fr system is not due to stronger electrostatic interactions. The nonelectrostatic contribution to ΔG at 2°C is calculated from the value at 1.0 M salt (9, 14) and is -9.3 Kcal mole $^{-1}$ for fr and -8.2 Kcal mole^{-1} for R17.

The K_a between fr coat protein and fragments 2 and 2-3 were measured as a

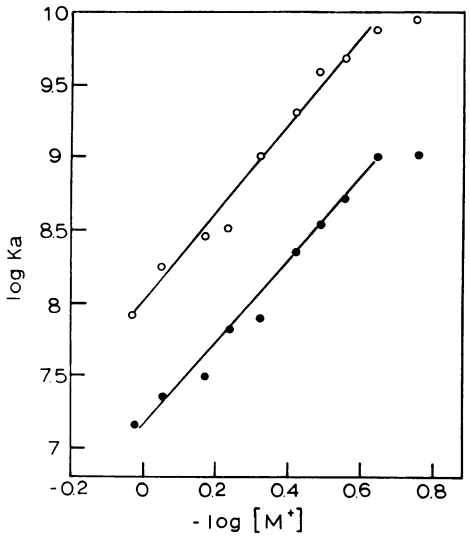

Figure 3: Ionic strength dependence of K_a. Protein excess binding curves with fragment 3 were obtained at 2°C in TMK buffer with KCl added to the indicated $[M^+]$. fr coat protein (○), R17 coat protein (●).

function of temperature. The ΔH for the RNA-protein interaction can be deduced from the slope of the van't Hoff plot. In Table 2, the thermodynamic parameters of fr coat protein binding to 2 and 2-3 are compared to those obtained previously for R17 coat protein binding to 2 and 2-1. Surprisingly, the tighter binding of the fr system is achieved by a more unfavorable entropy being offset by an even more favorable enthalpy. It is also interesting to

Table 2 Thermodynamic Parameters for Coat Protein Interaction with RNA Fragments at 2 °C.

coat protein	fragment	ΔG Kcal/mole	ΔH Kcal/mole	ΔS cal/mol-deg
fr	2	-12.4	-23.6	-40.7
fr	2-3	-11.9	-23	-40.4
R17+	2	-11	-19	-29
R17+	2-1	-13.7	-22	-30.2

+Data from references 9 and 13.

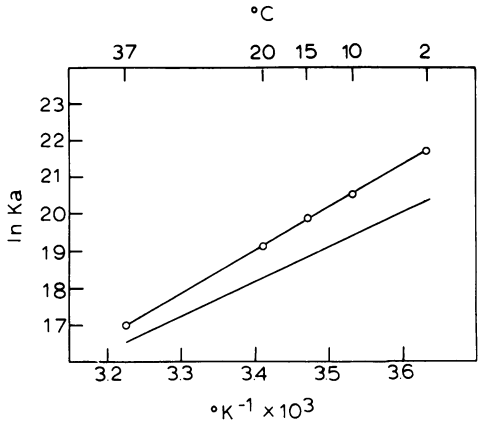

Figure 4: Temperature dependence of K_a. Protein excess binding curves were obtained in TMK buffer at different temperatures. fr coat protein to fragment 2-3 (o); The line indicates R17 coat protein binding to fragment 2 taken from reference 9.

note that as a result of the different ΔH values, the tighter binding observed with the fr system at 0°C is much less at 37°C (Figure 4).

Previous chemical modification studies showed the Cys_{46} of MS2 coat protein was essential for operator binding (15). In addition, H_2O_2 will inactivate the R17 coat protein by forming an intramolecular Cys_{46}-Cys_{101} disulfide bond (Hillbrand & Uhlenbeck, unpublished result). Thus for R17, Cys_{46} is thought to be the best candidate to form a putative transient covalent bond to the -5 residue of the operator RNA (12). Since fr coat protein also has cysteines at 46 and 101, it was tested for inactivation by H_2O_2. Under conditions where R17 coat protein is inactivated to greater than 99%, fr coat protein is inactivated by 95%. Thus it is likely that Cys_{46} in fr coat protein is also essential for the coat protein-operator interaction. However, the lower sensitivity of fr coat protein to H_2O_2 suggests that the structures of the fr and the R17 coat proteins may be slightly different in the neighborhood of Cys_{46}.

Mixed Capsid formation

From the above studies it is clear that the RNA binding surfaces of both fr and R17 coat proteins are very similar. In order to investigate whether the protein-protein contact surfaces of these two coat proteins are similar as well, capsid assembly reactions were performed by incubating a long operator RNA (fragment 11) and high concentrations of fr and R17 coat proteins. As

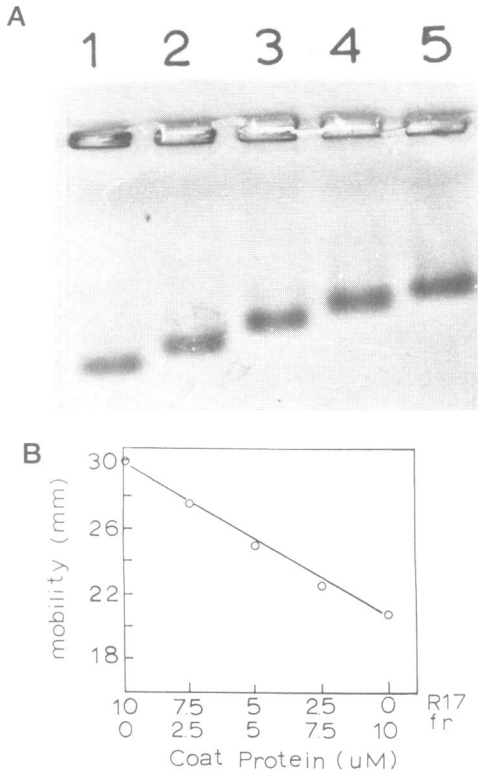

Figure 5: A: Agarose gel electrophoresis of the products at the capsid assembly reactions with fragment 11 and 10 μM R17 coat protein (lane 1), 10 μM fr coat protein (lane 5) or 7.5:2.5, 5:5, and 2.5:7.5 mixtures of the two (lanes 2-4); B: Mobility of reassembled capsids vs. the ratio of R17 to fr coat protein concentrations.

shown in figure 5A both proteins are able to form a virus-like capsid (16). As expected from the relative mobilities of the viruses (E. Gren personal communication), the fr capsid and R17 capsid have different mobilities on agarose gels. Also shown in Figure 5A is that when two coat proteins were mixed together in the capsid assembly reaction, capsids with intermediate mobilities were observed. Since there is a linear relationship between the mobility of the capsid and the input ratio of coat proteins (Fig. 5B), it appears that there is no preference for homologous interaction. This suggests that the protein-protein contact surfaces of fr and R17 coat proteins are very similar.

DISCUSSION

Despite the sequence differences between fr and R17 coat proteins, they recognize their operators in virtually the same way. The structure of the characteristic hairpin must be maintained by the base paired stem, but the sequence of the base pairs are not important. The identity of four of the single strand nucleotides (Pu_{-10}, A_{-7}, Py_{-5}, and A_{-4}) are essential for protein binding and their relative position with respect to one another must be maintained. Other single stranded residues must be there, but their sequence can vary. This similarity in the general rules for operator recognition by the two proteins is not only qualitative, but quantitative. When one of the essential nucleotides is changed to another nucleotide, the effect on K_a is the same for both proteins. This suggests that the contacts formed between the protein and RNA are nearly identical in the two cases.

The greatest difference between the fr and R17 systems is that fr coat protein binds every RNA about ten fold tighter. This tighter binding is a result of the non-electrostatic component of the RNA-protein interaction. One of the sites in the operator which may be responsible for the tighter binding is in the neighborhood of the bulged A residue since fr coat protein binds about three fold less well than expected at that site. An additional site may be responsible for the other factor of three in K_a, but it was not identified by the substitution experiments.

It is interesting to note that at the two positions (-17 and -6) where the two operators differ in nucleotide sequence, the proteins behave identically to nucleotide substitution. The sequence of position -17 is irrelevant for binding of both proteins. At position -6, both proteins prefer U over A, but the fr operator contains the slightly weaker A residue in order to partially compensate for the tighter overall binding. Thus the simple model that one or more of the 17 amino acid substitutions in fr coat protein are interacting directly with A_{-6} is not correct. Instead, the amino acids near position -6 are likely to be the same for both proteins and some of the fr substitutions are responsible for the tighter overall binding.

The observation that fr and R17 coat proteins can assemble together into capsids without apparent preference suggests that the several different coat protein-coat protein contacts involved in phage assembly are also very similar in the two systems. We have not been successful in demonstrating hybrid viruses in mixed infections, but this may well be the result of the tighter fr-RNA interaction preventing R17 from replicating within the same cell. The

similarity in the protein-protein and protein-RNA binding surfaces suggests that many of the amino acid substitutions are functionally silent and protrude into the solvent on the outer surface of the virus.

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