Selection and characterization of RNA aptamers to the RNA-dependent RNA polymerase from foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease virus causes a highly contagious disease of agricultural livestock and is of enormous economic importance. Replication of the RNA genome of the virus, via negative strand intermediates, involves an RNA-dependent RNA polymerase (3Dpol). RNA aptamers specific to this enzyme have been selected and characterized. Some of these molecules inhibit enzymatic activity *in vitro*, with IC_{50} values of <20 nM and K_i values of 18–75 nM. Two of these show similarity, both with each other and with regions of the viral genome. Furthermore, truncated versions of one of the aptamers have been used to define the parts of the molecule responsible for its inhibitory activity.

Keywords: aptamer; RNA polymerase; FMDV; *in vitro* selection; SELEX

INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most important diseases of domestic livestock, affecting a wide range of species, especially cloven hoofed animals. Although not normally fatal, the disease is one of the most highly contagious known. It spreads rapidly and is estimated to result in a 25% loss in productivity if not controlled (Brooksby 1982). The causative agent, foot and mouth disease virus (FMDV) belongs to the family *Picornaviridae*, which includes many human and animal pathogens, including poliovirus (PV) and rhinovirus (the main causative agent of the common cold). FMDV is highly variable. There are seven distinct serotypes, and each of these comprises a wide range of strains. In common with all picornaviruses, the FMDV genome is a single-stranded, positive-sense RNA molecule of ~8.5 kb comprising long 5' and 3' untranslated regions (UTRs) flanking a single open reading frame. The 5' UTR is exceptionally long, ~1.2 kb in length, and includes highly structured domains (Pilipenko et al. 1989; Mason et al. 2002, 2003). The

domain at the 5' terminus is ~400 nucleotides (nt) in length and is predicted to form an imperfect hairpin structure (Newton et al. 1985). This is known as the S fragment and is separated from the remainder of the genome by a long (150–250 nt) poly-cytidylic acid tract. This, in turn, is followed by a region of pseudoknot repeats of unknown function (Clarke et al. 1987), a *cis*-acting replication element (cre) necessary for the uridylation of the peptide primer (VPg) for RNA replication (Mason et al. 2002; Tiley et al. 2003), and finally an internal ribosome entry site (IRES) important for the initiation of translation (Belsham and Brangwyn 1990; Kuhn et al. 1990). By analogy with PV, the S fragment is likely to be important in the initiation and control of RNA replication. The polyprotein product of the open reading frame is initially processed into four regions: the L proteinase, P1 to 2A, 2BC, and P3. The latter three are further processed by virus-encoded proteases into the mature protein products. The structural proteins are derived from the N-terminal region (P1), and the proteins involved in viral replication occupy the C-terminal two thirds of the precursor polyprotein (P2, P3). The RNA-dependent RNA polymerase (RdRp), known as 3Dpol, responsible for replication of the genome (via negative strand intermediates), is located at the C-terminus of the polyprotein. Synthesis of the viral RNA is initiated by VPg, which undergoes post-translational uridylation by 3Dpol in conjunction with the precursor

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protein, 3CD, in the presence of cre (Nayak et al. 2005). cre is known to contain an essential AAACA motif; however, the details of the uridylation and priming mechanisms are poorly understood, and although these are common to all picornaviruses, there are a number of details that are unique to FMDV. In particular, the FMDV genome encodes three tandem copies of VPg, in contrast to the single copy found in the genomes of all other picornaviruses (Forss and Schaller 1982). These sequence data supported earlier biochemical evidence for the presence of multiple VPgs on virion RNA (King et al. 1980). Together, these studies showed that the three encoded VPgs are used with equal frequency in RNA replication. It is tempting to speculate that this repetition of the VPg region of the genome is related to the rapid replication of FMDV compared to other picornaviruses.

The atomic structures of 3Dpol of FMDV in the apo form and in complex with a synthetic primer/template have been solved at resolutions of 1.9 Å and 3 Å, respectively (Ferrer-Orta et al. 2004). This represents the first complete structure of a picornavirus polymerase and has the “palm, thumb, and fingertips” topology seen in other RdRps (O'Reilly and Kao 1998). Recently, the structure of 3Dpol complexed with the VPg primer peptide has also been published (Ferrer-Orta et al. 2006). Although these FMDV structures have significantly advanced our understanding of the properties of the polymerase, there are still many aspects of its function in replication or in the uridylation of VPg that are not fully understood. In an attempt to address some of these questions, we have selected specific molecular probes, RNA aptamers, as tools to dissect the functions of 3Dpol.

Aptamers are small single-stranded nucleic acid molecules that exhibit some of the characteristics of both antibodies and siRNAs. They share a number of potential applications with monoclonal antibodies but may be selected to have even higher affinities and specificities. In addition, they can be modified to increase their stability and to facilitate delivery into cells (for reviews, see Blank and Blind 2005; Bunka and Stockley 2006). They are, therefore, useful as probes to define binding sites for the design of small-molecule inhibitory compounds or in diagnostics (Brody et al. 1999; Collett et al. 2005; Yamamoto-Fujita and Kumar 2005; Bunka and Stockley 2006). As aptamers can be selected to directly inhibit an interaction, for example, between enzymes and their substrates or receptors and their ligands, it is clear that they could also have potential as therapeutics. A recent exciting development in this field has been the aptamer Macugen. This binds to vascular endothelial growth factor and is in clinical use for the treatment of age-related macular degeneration (Lee et al. 2005; Ng et al. 2006).

Aptamers are isolated using an *in vitro* selection technique, referred to as SELEX (systematic evolution of ligands by exponential enrichment) (Tuerk and Gold 1990;

Ellington and Szostak 1990) involving iterative rounds of target binding and preferential amplification. The technique can be applied to almost any target, and there are ~3000 aptamer sequences currently in the literature (<http://aptamer.icmb.utexas.edu>; Lee et al. 2004). However, targets from relatively few viruses are represented. Examples include aptamers to HIV Tat and reverse transcriptase (Ye et al. 1996; Nickens et al. 2003), hepatitis C virus (HCV) polymerase (see Biroccio et al. 2002; Vo et al. 2003) and NS3 (Nishikawa et al. 2003, 2004), influenza hemagglutinin (Jeon et al. 2004; Misono and Kumar 2005; Gopinath et al. 2006), and bacteriophage MS2 coat protein (Convery et al. 1998; Hirao et al. 1998; Rowsell et al. 1998; Shtatland et al. 2000). In this article, we report the identification and characterization of the first aptamers to any protein from FMDV; the 3Dpol enzyme (serotype C). Three of these RNA molecules display inhibitory activity in an *in vitro* UTP incorporation assay.

RESULTS

Aptamers with similarity to the viral genome

Lysine residues on the surface of 3Dpol were chemically biotinylated, allowing the enzyme to be immobilized onto the surface of streptavidin-coated magnetic beads. Fifteen rounds of selection were undertaken using a degenerate library of $\sim 10^{15}$ sequences, consisting of an N_{30} random region flanked by primer sequences for amplification and *in vitro* transcription. The SELEX procedure was carried out on a Biomek 2000 automated work station using protocols similar to those previously described by the Ellington Laboratory (Cox et al. 1998; Cox and Ellington 2001). As expected, the selected pool displayed increased affinity for 3Dpol compared with the naive RNA population as assessed by electrophoretic mobility shift assays (data not shown). Fifty-two cDNA clones were prepared from the round 15 selected pool, of which 25 were selected at random for detailed analysis and sequencing (Fig. 1A). Selection resulted in some of the aptamers incorporating N_{30} regions smaller than the original 30 nucleotides. The greatest reductions were seen with aptamers F4, F6, F12, and F38. Some minor mutations in the flanking sequences were also observed.

Sequence similarity was determined by using the GeneBee Multiple Alignment program (http://www.genebee.msu.su/services/malign_reduced.html). Two of the aptamers (referred to as F14 and F17) were identical and contained sequence motifs with significant similarity to sequences within the positive-sense viral genome, as did eight of the others (Fig. 1B). The remainder exhibited little similarity. The selected aptamers generally shared similarity with viral sequences clustered at the 5' UTR, especially in the first 364 nucleotides, corresponding to the S fragment. Similarity was also seen with the reverse complement of the genome

F6
GGGAAUGGAUCCACAUCUACGAAUUCCGUAAUUACCGUUUCACUGCAGACUUGACGAAGCUU

F7
GGGAAUGGAUCCACAUCUACGAAUUCAAUGGCGUUUCGAGUCAAUUCACUGCAGACAUUGAAGCUU

F9
GGGAAUGGAUCCACAUCUACGAAUUCUCUUUGGCUAGGGGCGCUUAUCCAUGUUUCACUGCAGACUUGACGAAGCUU

F10
GGGAAUGGAUCCACAUCUACGAAUUCCCUCCAAUCGCUGCGCGGCCUCCCCCAGUGGUUACACUGCAGACUUGACGAAGCUU

F12
GGGAAUGGAUCCACAUCUACGAAUUGGCGUGAGUUACCCGUGAUUCACUGCAGACUUGACGAAGCUU

F14
GGGAAUGGAUCCACAUCUACGAAUUCUCGUCCUUUCGCGUUUCCGGGUGGGGUAGAUUACACUGCAGACUUGACGAAGCUU

F17
GGGAAUGGAUCCACAUCUACGAAUUCUCGUCCUUUCGCGUUUCCGGGUGGGGUAGAUUACACUGCAGACUUGACGAAGCUU

F18
GGGAAUGGAUCCACAUCUACGAAUUCGAGAAUUUACCCGCGACUCCCCUUCAGCAGUUUUACACUGCAGACUUGACGAAGCUU

F32
GGGAAUGGAUCCACAUCUACAAAUUC GUGAAACGAAAAACCCCAGUGGCUGGGUGGUUUUACCGGAAACUGGACAAAGCUA

F33
GGGAAUGGAUCCACAUCUACAAAUUC UCGUCCUUUCGGGUUUCCGGGGGGGGUAAAAUCAUUGGAAAGUUGACAAAGCUA

F34
GGGAAUGGAUCCACAUCUACGAAUUCUGAGCUUAGGGGGGCAAAAAUAGCAGUUUGACAAACCUA

F35
F35GGGAAUGGAUCCACAUCUACGAAUUCUUUACCCAAAACGGAGCUGUGUUCCUUACCAUUACACUGCAAACUUGACAAAGCUA

F36
GGGAAUGGAUCCACAUCUACGAAUUCAAAGGGGGUUAGGUAAAAGGAGUCGUUUUACACUGCAAACUUGACAAAGCUU

F37
GGGAAUGGAUCCACAUCUACGAAUUCCCGAAACUUUUCCUUAGCCGACCACAGCCUUUCACUGCAAACUUGACAAACCUA

F38
GGGAAUGGAUCCACAUCUACAAAUUCCCUUAUAUAAACCCGUCCACGCCCUUCGAAGCAGUUCAGACGUCACUU

F40
GGGAAUGGAUCCACAUCUACAAAUUCACTAUUGUGACCAGUAGCTCGUAUGG UUAACUGCAAACUUGACAAAGCUA

F41
GGGAAUGGAUCCACAUCUACAAAUUCCCUCACCUAAUUUUAUCCUCCCCGGCGUACGGUUUAACUGCAAACUUGACAAACCUA

F42
GGGAAUGGAUCCACAUCUACAAAUUCAAAGGGCGAGUUAUCAGGAGGGAAAAGGCAUUUAUGGAAACUUGAAGAAACUU

F44
GGGAAUGGAUCCACAUCUACAAAUUCAAAAAGUUUUACCCGUUUUGCUAAAAAGUUUAACUGCAAACUUGACAAAGCUA

F45
GGGAAUGGAUCCACAUCUACAAAUUCCCAAACGUUUCCGUAGCCGACCACAGCCUUUCACUGCAAACUUGACAAACCUA

F46
GGGAAUGGAUCCACAUCUACAAAUUCGCGCCUGUAAUCUGUGUCUAAAAAACGUGUUUUACUGCAAACUUGACAAACCUA

F47
GGGAAUGGAUCCACAUCUACAAAUUCGUUAAACAGAAAAACUCUCAGUUGCGGGUUGUUUUACUGCAAACUUGACAAAGCUA

F49
GGGAAUGGAUCCACAUCUACAAAUUCCGCCCGUAAAGUUCCCCAAUUACCCGUGCAUUCACUGCAAACUUGACAAACCUA

F51
GGGAAUGGAUCCACAUCUACGAAUUCCCGGAAUUUUCCUUAGCCGACACAGCCUUUCACUGCAAACUUGACAAACCUA

F52
GGGAAUGGAUCCACAUCUACGAAUUCCUUACCCGUAGACCCGAGUUGCUGAGUUUGCGAAGCAGUUCAGCGUCACU

RNA	FMDV-C Start		RNA Sequence	
F47		5'	GUUAAACAGAAAGCUCAGUUGCUGGGUUGU	3'
F52		5'	CUUACCCGUGAACCGCAGUUGCUGAGUUG	3'
FMDV-C	28	5'	CUAGCAUCCAGAGAGAGUCCUGCGGUGC	3'
F12		5'	GCUGAGUUCACCGUGUA	3'
F18		5'	GAGAAUUCACCGGACUCCCCU	3'
FMDV-C	107	5'	CUAUCGUCACCCACCAUCAGCU	3'
F12		5'	GUGUGUUCACCGUUA	3'
F18		5'	GAGAAUUCACCGGACUCCCCUUCGACUGU	3'
FMDV-C	124	5'	ACUCACGGACCCCGUGCCAUUUUAGCUGG	3'
F14		5'	UCGUCUUUCGCGUUCGCGUGGGGUAGA	3'
F17		5'	UCGUCUUUCGCGUUCGCGUGGGGUAGA	3'
F33		5'	UCGUCUUUCGCGUUCGCGUGGGGUAGA	3'
FMDV-C	232	5'	CGCGUCUUCGGGCACUCGUGUGGGGCGCG	3'
F47		5'	GUUAAACAGAAAGCUCAGUUGCUGGGUUGU	3'
F52		5'	CUUACCCGUGAACCGCAGUUGCUGAGUUG	3'
FMDV-C	507	5'	CUUGAAGUAAACAGACCAUCGACACAGUU	3'
F14		5'	UCGUCUUUCGCGUUCGCGUGGGGUAGA	3'
F17		5'	UCGUCUUUCGCGUUCGCGUGGGGUAGA	3'
FMDV-C	569	5'	CGCAAGAAACCGCGCGUUCUGAGGAGAA	3'
F37		5'	CGAAGAUUUUCUAAGCCGACAGAGCCU	3'
F45		5'	CCCAACGCUUUCGUAACCGGACAGAGCCU	3'
F51		5'	CGGAGAUUUUCUUAAGCCGACAGAGCCU	3'
FMDV-C	859	5'	CAACACUUUUCUGAAGACCAACUUUAG	3'

RNA	FMDV-C Start	RNA Sequence	
3'UTR			
F10	3'	CUCCAAUGGUGGCGGGCCUCCCCAGUGG	5'
F18	3'	GAGAAUUGACCGCGACUCCCUUUCGACUGU	5'
FMDV-C	1	AAUUUUCGCCCGCAUCCCGACAGUGGGGAU	5'
F35	3'	UUUACCCAAACGGAGCUGUGUUCUUAACCA	5'
F45	3'	CCAAACGUGUCCGUACCGGACACGAGCCU	5'
FMDV-C	41	GGGACCCAAACGUGAGGUGUGAAUGCACACGGG	5'
F33	3'	UCGUCCUUUCGGGUUCCGCGGGGUGUAAA	5'
F34	3'	UGAGUUUACGGGGGCAAAAACUAAAGC	5'
FMDV-C	261	GCAAGCUGCGAGGUGGCCGAGGGGACACGCGUUGAU	5'
F36	3'	AAGGGGGUUAAGGUAAAAGGAGUCUGUUU	5'
F42	3'	AAAGGGCGAGUUUAUCAGGAGGGGAAGGCAU	5'
FMDV-C	677	GCCGUGGAAAAGCGAAUAUGUUGAUGACUGAUGU	5'
5'UTR			
F14	3'	UCGUCUUUCGUGUUCGCGGUGGGGUAAG	5'
F17	3'	UCGUCUUUCGUGUUCGCGGUGGGGUAAG	5'
F33	3'	UCGUCUUUCGUGUUCGCGGGGGGGUAAA	5'
FMDV-C	58	CACUUUCGGGCGGCGGCGGAAAAGCGGA	5'
F36	3'	AAGGGGGUUUAGGUAAAAGCUGCUUUU	5'
F42	3'	AAAGGGCGAGUUUAUCAGGAGGGGAAGGCAU	5'
FMDV-C	71	AAAGGGCGAGGAGTATAGGAAAAAAAAGAA	5'

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to regions that correspond to the 3' and 5' UTRs of the negative strand during replication (Fig. 1C). Aptamers F14/17, F18, and F33 share similarity with both the genome and the reverse complement. Little similarity was found between any of the aptamers and the coding region or the 3' end of the genome. It is possible that the similarity between some of the aptamers and regions of the 5' end of the genome (and possibly the 3' and 5' ends of the negative strand) may represent preferred genomic binding sites of the enzyme during replication. Interestingly, runs of four or more As were seen in six aptamers (F32, F34, F36, F44, F46, and F47), and an AAAC motif was present in nine of the aptamers sequenced (F32, F34, F35, F37, F38, F44, F45, F46, and F47), the motif being AAACA for one of the aptamers (F44). This shares similarity with the conserved AAACA sequence of cre (Mason et al. 2002).

Inhibition of polymerase function

To investigate the ability of the anti-3Dpol aptamers to inhibit RNA synthesis, we employed polymerase activity assays that measured the incorporation of α - 32 P UTP into an RNA product. The rate of incorporation is linear over a 2-h period (Fig. 2A). For the inhibition experiments, the polymerase was preincubated with selected aptamers at a range of concentrations for 15 min, prior to assessing the ability of each aptamer to reduce polymerase activity after 1 h. As a negative control, identical assays were undertaken using the unselected N₃₀ RNA pool. In previous studies using HCV polymerase, aptamers were selected that either displayed similarity with the viral genome (Biroccio et al. 2002) or showed no significant similarity (Vo et al. 2003). In similar polymerase inhibition assays, both classes were found to inhibit the function of the polymerase in vitro. It is likely that the two selections resulted in aptamers that recognize different sites (apatoxes) on the target molecule

and that may inhibit the enzyme by different mechanisms. In our study 22 aptamers were tested. These included all of the aptamers with similarity to the genome and negative strand listed in Figure 1, B and C (with the exception of F36), as well as eight others selected at random. Most of the aptamers had little effect on the enzyme, reducing the activity by 0%–23% at the highest concentration tested (100 nM). However, three were found to have more dramatic effects, resulting in 68%–93% reduction in activity. The data presented in Figure 2B and Table 1 show that aptamers F38, F47, and F52 inhibit the enzyme at IC₅₀ of 15.8 ± 3.4 nM, 10.6 ± 2.1 nM, and 16.4 ± 3.1 nM, respectively. As a negative control, identical assays were undertaken using the naive RNA pool, which showed no inhibitory activity. F47 and F52 share 76% sequence identity in their N₃₀ regions. They also share similarity with sections of the viral genome: 53% and 62% for F47 and F52, respectively, with the S fragment (nucleotides 28–57) and 37% and 34%, respectively, with a second region in the 5' UTR (nucleotides 507–536, 5' to the pseudoknot region; Fig. 1). The third inhibitory aptamer (F38) had little sequence similarity with the genome (positive or negative strand) or with the other aptamers. The specificity of the inhibitory aptamers (F38, F47, and F52) was investigated in a similar assay with the related polymerase from PV. It was found that the aptamers had no effect on the activity of this enzyme (Fig. 2C). The polymerases from FMDV-C and PV share 29% overall sequence identity, with 69% in the regions in contact with the primer/template and incoming NTP. The overall architecture of FMDV 3Dpol is similar to that seen in the crystal structure of PV polymerase (Thompson and Peersen 2004). In both structures, there are extensive interactions between thumb and finger domains, fully enclosing the active site. Furthermore, the shape and size of the central cavity (which accommodates primer/template) are almost identical (Ferrer-Orta et al. 2006).

FIGURE 2. (A) Incorporation of α - 32 P UTP by 3Dpol in the presence of a primer/template is linear over a 2-h period; 4 nM FMDV 3Dpol was incubated with a α - 32 P UTP substrate and primer/template. (B) Aptamers F38 (●), F47 (▲), and F52 (▼), but not the naive RNA pool (■), inhibit FMDV 3Dpol; 4 nM was incubated with a α - 32 P UTP substrate, primer/template and increasing concentrations of aptamer RNA. The amount of α - 32 P UTP incorporated into the polymer was measured, and the activity was calculated as a percentage of the activity of 3Dpol in the absence of aptamer. (C) The same aptamers do not inhibit PV 3Dpol; 10 nM PV 3Dpol was incubated with a α - 32 P UTP substrate, primer/template, and increasing concentrations of RNA as in B.

TABLE 1. IC_{50} and K_{Dapp} values for aptamer:3Dpol interaction derived from polymerase activity and affinity assays

	IC_{50} (nM)	K_{Dapp} (nM)
F38	15.8 ± 3.4	460
F47	10.6 ± 2.1	375 ± 46
F52	16.4 ± 3.1	625
F47tr	10.2 ± 1.6	325

Affinity of aptamers for 3Dpol

Binding affinities of the RNA aptamers for the 3Dpol protein were measured using a bead-based immobilization/precipitation assay that we have developed (Robinson et al. 2006), similar to a method described previously (Bae et al. 2002; Hwang et al. 2004). Increasing amounts of biotinylated 3Dpol were immobilized onto streptavidin beads before incubation with α - ^{32}P UTP radiolabeled RNA aptamers. The amount of aptamer bound was quantified by measuring RNA band intensity after electrophoresis and autoradiography of RNA eluted from the beads. By comparison with a maximum binding value, data are represented as a percentage of RNA bound, allowing calculation of the apparent dissociation constant (K_{Dapp}) (Fig. 3). K_{Dapp} for the protein with the naive RNA pool control was estimated to be 4 μ M, whereas for aptamers F38, F47, and F52, K_{Dapp} = 460 nM, 375 ± 46 nM, and 625 nM, respectively.

Definition of aptamer minimal sequences

The aptamer sequences described above are 75–80 nt in length, as in addition to the N_{30} random region, they included flanking primer sequences (Fig. 1). Any similarity to the viral genome was contained within the random region. Therefore, shortened templates were designed that encompassed the N_{30} regions and T7 RNA promoter site only. The truncated aptamers (F38tr, F47tr, and F52tr) were synthesized by in vitro transcription. As two Gs were added at the 5' ends of the N_{30} region templates to facilitate transcription, this resulted in RNA molecules of 27–32 nt in length. A control RNA (referred to as viraltr) was also produced. This corre-

sponded to nucleotides 28–57 of the FMDV-C genome to which F47 and F52 share similarity (Fig. 4A). Truncation of aptamer F47 in this way did not significantly affect its binding affinity for 3Dpol (K_{Dapp} = 325 nM for F47tr, compared with 375 ± 46 nM for full-length aptamer F47) or its ability to inhibit polymerase activity (IC_{50} = 10.2 ± 1.6 and 10.6 ± 2.1 nM for F47tr and F47, respectively) (Table 1; Fig. 4B). However, truncation of aptamers F38 and F52 resulted in the loss of inhibitory activity, despite the fact that F52tr shares 77% sequence similarity with F47tr. The viraltr RNA displayed no inhibitory activity in the assay.

Mfold secondary structure predictions (Zuker 2003) suggest that both aptamers F47 and F47tr contain an 18 nucleotide stem-loop that was not predicted in the non-inhibitory viraltr sequence. To investigate the possible importance of this motif in recognition and inhibition of the 3Dpol enzyme, an oligonucleotide corresponding to this stem-loop was designed and synthesized by phosphoramidite chemistry and purified by high-pressure liquid

FIGURE 3. Binding affinities of aptamers F38, F47, and F52 and the naive RNA pool (C) for the FMDV 3Dpol protein. Radiolabeled RNA aptamers (10 nM) were incubated with increasing amounts of biotinylated 3Dpol protein. Bound RNA was eluted and electrophoresis undertaken on a 12% denaturing polyacrylamide gel, quantitated by autoradiography. Images of these gels are shown, with the concentration of 3Dpol used indicated for each lane. The percentage of input RNA bound to 3Dpol was calculated by determining the fraction of radioactivity present in each lane. The concentration of protein at which maximum binding of RNA to 3Dpol was observed was seen at 2 μ M with aptamers F38, F47, and F52, and 5 μ M for the control RNA (the naive RNA pool). The values measured have been normalized to these respective amounts.

FIGURE 4. Definition of aptamer sequences. (A) Sequences of truncated aptamers that corresponded to the N₃₀ region of each full-length aptamer and two additional 5' G residues. (B) Truncated aptamers were assayed for their ability to inhibit the catalytic activity of 3Dpol in order to assess which parts of the sequences are important for this inhibition. Upon truncation, aptamers F38tr (●) and F52tr (▼) no longer inhibited 3Dpol, whereas the activity of aptamer F47tr (▲) was unaffected, compared with a viraltr RNA control (nucleotides 28–57 of the FMDV-C genome) see A (■).

chromatography (HPLC) (Fig. 5A). The ability of this synthetic RNA to inhibit the activity of 3Dpol was measured, as described previously. The data from these α -³²P UTP incorporation assays (Fig. 5B) show that although the stem-loop does inhibit the 3Dpol, it was less effective than either the full-length or truncated 47 aptamer, reaching only a 30% reduction in polymerase activity (in the presence of 100 nM aptamer). An extended RNA stem-loop incorporating two additional GC base pairs at the 5' and 3' ends, designed to increase the T_m, gave a similar result (32% inhibition).

Mode of aptamer inhibition

The mode of inhibition by aptamers F38, F47, F52, and F47tr was investigated by carrying out α -³²P UTP incorporation assays using varying concentrations of primer/template similar to those described above, but in the presence of increasing amounts of aptamer RNA. This allowed comparison of calculated values of V_{max} for the reaction, K_m for the primer/template, and K_i. The data, illustrated in Figure 6 and Table 2, show that the presence of tRNA has little effect on either V_{max} or K_m. For all of the aptamers tested, however, V_{max} values were reduced. V_{max} in the absence of aptamer = 1664 ± 92 nmol/h. In the presence of 10 nM or 100 nM of F38, this decreased to 1131 ± 75 and 671 ± 59 nmol/h, respectively. A similar

decrease was seen for F47 (1072 ± 31 and 407 ± 20 nmol/h), F47tr (976 ± 67 and 322 ± 9 nmol/h), and F52 (1467 ± 103 and 814 ± 103 nmol/h) with 10 nM or 100 nM aptamer, respectively. The pattern for K_m was more complicated. In the absence of aptamer, K_m = 346 ± 40 nM. In the presence of aptamer F38 at 10 and 100 nM, K_m = 334 ± 52 and 295 ± 60 nM, respectively. The lack of systematic variation in these values indicates that F38 inhibits the 3Dpol enzyme noncompetitively with respect to the primer/template RNA. More variation was seen with K_m for the other aptamers tested (508 ± 37 and 309 ± 39 nM for F47, 503 ± 93 and 215 ± 13 nM for F47tr, and 523 ± 88 and 443 ± 79 nM for F52 at 10 nM and 100 nM, respectively). Under these conditions, aptamer F47tr had the lowest K_i value (14 nM), followed by F47 (18 nM) F38 (21 nM), and F52 (75 nM). K_i values are related to K_D, and given that the lower the K_i, the more effective the inhibitor, this result confirms previous data indicating that F47 and F47tr are the best inhibitors.

DISCUSSION

We report the selection and characterization of RNA aptamers to the 3D RdRp, a key part of the FMDV replication complex. This is the first report of aptamer selection to any protein of this economically important

FIGURE 5. (A) The predicted secondary structure of the stem-loop of aptamer F47, together with an extended stem-loop incorporating two additional GC base-pairs. (B) The stem-loop (▲) and the extended stem-loop (●) retain some ability to inhibit 3Dpol compared with data for the naïve RNA pool (■), showing an ability to reduce the catalytic activity of 3Dpol by ~30%.

FIGURE 6. Inhibition of the 3Dpol interaction by aptamers F47 (A), F52 (B), and F38 (C). 3Dpol (8 nm) was incubated with various concentrations of each aptamer (■) 0 nM; (●) 10 nM; (▲) 100 nM and increasing amounts of primer/template for 1 h. Kinetic parameters $V_{\max}$ and K_m were calculated using the Hill equation.

animal pathogen. We have characterized three aptamers (F38, F47, and F52) that inhibit the *in vitro* activity of the enzyme with IC_{50} values of 15.8 nM, 10.6 nM, and 16.4 nM, respectively. One aptamer retains this inhibitory ability when truncated to a 32mer (F47tr) with an IC_{50} of 10.2 nM.

Although they are efficient inhibitors, the aptamers show lower affinity for the enzyme than expected, with K_{Dapp} values of 325–625 nM. This is likely to be due to the technique used to measure K_{Dapp} . Immobilization of 3Dpol to streptavidin beads involved biotinylation of surface lysines. Together with the steric effects of immobilization, this could result in a loss of aptatopes and therefore produce a higher K_{Dapp} than expected. Although the same strategy was employed to immobilize the protein as a selection target, it seems likely that the level of biotinylation was different in the two experiments. Unfortunately, there was an insufficient amount of biotinylated protein available to establish the extent of derivatization by mass spectrometry. The trend seen in the affinity assays, however, does mirror that of the inhibition studies, since aptamers F47 and F47tr have higher affinities (K_{Dapp} = 325 and 375 nM) for 3Dpol than F38 and F52 (K_{Dapp} = 460 and 625 nM). A similar pattern is seen with the K_i values. These are related to K_D and are lower for aptamers F47 and F47tr (18 and 14 nM) than for aptamers F38 and F52 (21 and 75 nM, respectively). Furthermore, it therefore appears that the K_i data more accurately reflect the affinity of the aptamer–apatope interaction. Further truncation of F47tr into an 18-nt stem–loop resulted in a substantial (but not total) loss of inhibition. This truncation involved the removal of the 5' end of the molecule, and it is therefore likely that this region contributes to 3Dpol inhibition. In collaboration with Nuria Verdaguer (Barcelona), we have undertaken co-crystallization studies of F47tr with 3Dpol. From the difference maps, electron density was well defined, allowing the positioning of a fragment of single-stranded RNA (six of the 32 bases) in the central cavity. Additional density was also visible, accommodating two or three aptamer bases loosely contacting the fingertip region. Both regions of

density were compatible with the binding of single-stranded RNA. Although these observations are very preliminary, refinement of the complex is now in progress (N. Verdaguer, pers. comm.).

Similarity with the viral genome is not necessarily indicative of inhibitory activity. Aptamers F47tr and F52tr share 50% and 58% similarity with the S fragment, 34% and 32% to a second region within the 5' UTR, and 77% sequence identity with each other; however, only 47tr is inhibitory. Of the other aptamers characterized from the initial pool, several had similarities of up to 47% (in the N_{30} regions) with the viral genome (F14/F17 and F33), but none of these showed significant inhibition. The N_{30} region of F14/F17 also showed 47% similarity with the negative strand of the genome. Furthermore, the inhibitory aptamer F38 shows little genome similarity. This is suggestive that the inhibitory aptamers may recognize a range of 3Dpol apatopes. From the kinetic data, aptamer F38 appears to exhibit a noncompetitive mode of inhibition. The data for aptamers F52, F47, and F47tr are less clear. From the preliminary structural studies described above, it appears that F47tr binds both in the active site of the polymerase in

TABLE 2. $V_{\max}$, K_m , and K_i values for aptamer:3Dpol interaction

	$V_{\max}$ nmoles/hr	K_m nM	K_i nM
0 nM aptamer	1664 ± 92	346 ± 40	ND
10 nM F38	1131 ± 80	334 ± 52	21
100 nM F38	671 ± 59	295 ± 60	
10 nM F47	1072 ± 31	508 ± 37	18
100 nM F47	407 ± 20	309 ± 39	
10 nM F52	1467 ± 103	523 ± 88	75
100 nM F52	814 ± 103	443 ± 79	
10 nM F47tr	976 ± 67	503 ± 93	14
100 nM F47tr	322 ± 9	215 ± 13	
10 nM tRNA	1447 ± 32	365 ± 21	ND
100 nM tRNA	1408 ± 23	381 ± 41	

ND indicates not determined.

the central cavity and to a region distant from this, and therefore, the aptamer may inhibit both competitively and noncompetitively. From the electron density, however, it is impossible to distinguish between a model in which two RNA aptamers have bound to a single molecule of 3Dpol at the two sites or one in which a single RNA binds at both sites on the protein, but the intervening RNA sequence is too unstructured to give a signal. The similarity between aptamers F47 and F52 with regions in the 5' UTR could suggest preferred genomic binding sites for 3Dpol during replication. Furthermore, aptamers F47 and F47tr contain an AAAC motif, which is also present in eight others (F32, F34, F35, F37, F38, F44, F45, and F46). This shares similarity with the conserved AAACA sequence of cre (Mason et al. 2002) and may reflect cre–3Dpol interactions during uridylation of VPg as well as a preference by 3Dpol for binding AAAC sequences, which are distributed throughout the 5' UTR and the genome.

Aptamers F38, F47, and F52 are specific to FMDV and do not inhibit the related polymerase from PV. This is likely to reflect subtle differences in the structures of the two polymerases, and could be informative with respect to modeling the site of aptamer interaction. However, in order to fully understand replication in this virus and to probe the fine details of the interactions, it will be necessary to raise and characterize aptamers to other components of the FMDV replicase machinery for use in structural studies as well as in vitro and in vivo assays.

MATERIALS AND METHODS

In vitro selection

Selections against FMDV-C 3Dpol using a degenerate library of $\sim 10^{15}$ RNA sequences were undertaken using the following protocol.

The library (5'-GATAATACGACTCACTATAGGGAATGGATCCACACATCTACGA-N₃₀.TTTCAAGCAGTTCAGCGTCACTT-3') consisted of a 30-mer random region, flanked with conserved primer sequences for PCR amplification and a T7 polymerase promoter sequence (highlighted in bold) for in vitro transcription. The initial pool was generated by subjecting the DNA library to 15 cycles of PCR in a 50 μ L volume containing a final concentration of 5 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 μ M EDTA, 0.1 mM DTT, 5% glycerol, 0.1% Triton X-100, 1.5 mM MgCl₂, 10 nM template, 500 μ M of each dNTP, 1 unit (according to the manufacturer's instructions) of Taq DNA polymerase (Promega), and 1 μ M each of the upstream and downstream primers specific to the template: 5'-GATAATACGACTCACTATAGGGAATGGATCCACACATCTACGA-3', 5'-AAGCTTCGTCAAGTCTGCAGTGAA-3'.

C-His-tagged 3Dpol (2.4 nmol) (a gift from Esteban Domingo, Madrid) was biotinylated with EZ-Link Sulpho-NHS-LC-LC-Biotin reagent (Pierce). This allowed immobilization onto streptavidin-coated magnetic MyOne beads (DynaL Biotech) at 10 mg/mL in selection buffer (50 mM Tris-HCl at pH 7.5, 100 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM EDTA). As lysine residues may be involved in RNA binding, care was taken to ensure only partial biotinylation occurred. In order to ensure a low level of

biotinylation, the protein was incubated with the EZ-Link reagent for 30 min, rather than the 2 h recommended by the manufacturer. Binding to magnetic beads was confirmed spectrophotometrically by calculating the concentration of supernatant protein ($\lambda = 280$ nm) before and after incubation with the support beads. After PCR, the N₃₀ library was subjected to in vitro transcription, by incubating with 40 mM Tris-HCl (pH 7.9), 8 mM MgCl₂, 25 mM NaCl, 2 mM spermidine, and 500 μ M each of ATP, CTP, GTP, and UTP and 1.75 units of T7 RNA polymerase. Reactions were overlaid with mineral oil and incubated at 37°C for 1 h. The product of this transcription was incubated for 5 min at room temperature with 100 pmol of 3Dpol in selection buffer before magnetic separation of the immobilized protein and removal of supernatant unbound RNA. After washing twice with selection buffer, any RNA that was bound to 3Dpol was eluted by incubating the immobilized protein in pure water (Sigma) for 15 min at 96°C. This eluate was subjected to reverse transcription by first incubating with 1.25 μ M downstream primer, and 500 μ M of each dNTP for 15 min at 65°C, followed by incubation with 1.75 units Superscript II (Invitrogen) for 1 h at 37°C with a final concentration of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂. Five microliters of the reverse-transcription product were subjected to PCR before being used as the sequence pool for the next round of selection, of which 14 subsequent rounds were performed using a Biomek 2000 Automated Workstation (Beckman Coulter). Negative selection against biotinylated beads alone was carried out prior to rounds 1, 6, and 11 to reduce the likelihood of aptamer selection against the streptavidin beads.

RNA synthesis

In vitro transcription reactions were carried out in a 20 μ L reaction volume containing 40 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 10 mM NaCl, and 2 mM spermidine. The mixture also contained 500 μ M each of ATP, CTP, GTP, and UTP; 1.75 units of T7 RNA polymerase, according to the manufacturer's instructions (Promega); and 5 μ L DNA template from a PCR reaction. After incubation for 1 h at 37°C, RNA was subjected to acidified phenol:chloroform extraction and ethanol precipitation. The RNA was analyzed using a 12% (w/v) 19:1 acrylamide:bis-acrylamide denaturing polyacrylamide gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) and 7 M urea and confirmed to be a single band.

The 18- and 22-mer stem-loops derived from the aptamer F47 sequence were synthesized using solid-phase 2'-ACE phosphoramidite chemistry (Dharmacon Inc.) (Scaringe et al. 1998). Purification was achieved by HPLC on a DNAPac PA100 column (Dionex) eluting with a 0–1 M NaCl gradient at 65°C. Samples were desalted using a HiPrep 26/10 Desalting Column (Amersham Biosciences), eluting with H₂O (Murray et al. 1994).

Polymerase activity assays

Incorporation of α -³²P-labeled UTP into nascent transcripts by FMDV 3Dpol was investigated as an assay of polymerase activity. Assays were undertaken using 2 μ g Poly-(A) template (average length, 320) (Amersham), 2.4 μ M primer (oligo d(U)₁₅), and 200 μ M UTP; 5 μ Ci α -³²P-UTP; and a final concentration of 30 mM MOPS (pH 7.0), 25 mM NaCl, 5 mM Mg(CH₃COO)₂, in a volume of 46 μ L. The reaction was started by adding FMDV-C 3Dpol to

a final concentration of 4 nM. Samples were incubated at 30°C for up to 2 h for the time-course experiment and for 1 h for inhibition studies. Reactions were stopped by adding 10 μ L of 0.5 M EDTA. Ten microliters of each reaction were spotted onto separate DE-81 filters and washed with 200 mL 0.2 M Na_2HPO_4 five times, before washing with 500 mL dH_2O for 1 min and 100 mL 100% ethanol for a further minute. The filter squares were then air dried at room temperature for 1 h and the radioactivity quantified by scintillation counting. As a negative control, identical assays were undertaken using the naive RNA pool. Assays were performed in triplicate to allow the calculation of standard errors. Similar assays were also undertaken with PV 3Dpol (a gift from Jim Hogle, Harvard, Cambridge, MA) at 10 nM concentration.

Affinity measurements

Bead-based affinity assays were employed with individual aptamers to measure their affinity for the 3Dpol protein. These were similar to those employed previously to investigate aptamer affinity (Bae et al. 2002; Hwang et al. 2004). 3Dpol protein was biotinylated and immobilized onto streptavidin-coated magnetic beads (as above). Varying amounts of immobilized protein were incubated with 10 nM α - ^{32}P -UTP-labeled RNA, in 30 mM MOPS (pH 7.0), 25 mM NaCl, and 5 mM $\text{Mg}(\text{CH}_3\text{COO})_2$. After 15 min, the immobilized protein was pelleted and the supernatant, containing unbound RNA, was removed. RNA bound to the protein was eluted by adding water and heating to 96°C for 5 min before electrophoresis on a 12% (w/v) 19:1 acrylamide:bis-acrylamide polyacrylamide gel containing TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) and 7 M urea. The concentration of protein at which maximum RNA binding was observed was used as a standard for calculating the K_{Dapp} . After autoradiography, band intensity was quantified by using GeneSnap imaging software (Syngene). The percentage of RNA bound was calculated by using the concentration of protein that showed maximum RNA binding as a reference. This was plotted against concentration of 3Dpol, allowing calculation of the K_{Dapp} (Bae et al. 2002; Robinson et al. 2006). As a negative control, identical assays were undertaken using the naive RNA pool.

Kinetic assays

The mechanism of inhibition of each aptamer was determined with respect to the template/primer RNA. Inhibition assays were carried out under conditions as described above but with 8 nM 3Dpol and increasing concentrations of poly(A)/oligo(U) template/primer and RNA aptamer. After 1 h, the amount of α - ^{32}P UTP incorporated into the polymer was calculated, enabling the V_{max} of the reaction and K_{m} for the primer/template to be determined at various concentrations of each aptamer using the Hill equation (Equation 1). K_i values were calculated using Equation 2.

$$y = V_{\text{max}} \frac{x^n}{k^n + x^n} \quad (1)$$

$$V_{\text{max}} = \frac{V_{\text{max}}}{(1 + [I]/K_i)} \quad (2)$$

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