

# Probing RNA-Protein Interactions Using Pyrene-labeled Oligodeoxynucleotides: Q $\beta$ Replicase Efficiently Binds Small RNAs by Recognizing Pyrimidine Residues

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Binding of small RNAs by the RNA-dependent RNA polymerase of coliphage Q $\beta$  was studied utilizing a fluorometric assay. A DNA oligonucleotide probe of sequence 5'-d(TTTTTCC) was 5'-end-labeled with pyrene. In this construct, the proximal thymine residues efficiently quench the fluorophore emission in solution. Upon stoichiometric binding of one probe per polymerase molecule, the pyrene steady-state fluorescence increases by two orders of magnitude, the fluorescence anisotropy increases, and a long fluorescence lifetime component of 140 ns appears. With addition of replicable RNA, steady-state fluorescence decreases in a concentration dependent manner and the long lifetime component is lost. This observation most likely reflects displacement of the pyrene-labeled probe from the proposed nucleic acid binding site II of Q $\beta$  replicase. The effect was utilized to access binding affinities of different RNAs to this site in a reverse titration assay format. In 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, at 16°C, equilibrium dissociation constants for different template midi- and minivariant RNAs were calculated to be in the nanomolar range. In general, the minus and plus strands, concomitantly synthesized by Q $\beta$  replicase during replication, exhibited discriminative affinities, while their hybrid bound less efficiently than either of the single strands. Different non-replicable tRNAs also bound to the polymerase with comparable dissociation constants. By titration with DNA homo-oligonucleotides it was shown that the probed site on Q $\beta$  replicase does not require a 2' hydroxyl group for binding nucleic acids, but recognizes pyrimidine residues. Its interaction with thymine is lost in an A·T base-pair, while that with cytosine is retained after Watson-Crick base-pairing. These findings can explain the affinities of RNA-Q $\beta$  replicase interactions reported here and in earlier investigations. The sensitivity of the described fluorometric assay allows detection of RNA amplification by Q $\beta$  replicase in real-time.

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Abbreviations used: (+), plus strand; ds, double-stranded;  $K_d$ , equilibrium dissociation constant; MDV, midvariant RNA; MNV, minivariant RNA.

## Introduction

Q $\beta$  replicase is the RNA-dependent RNA polymerase responsible for replication of the single-stranded RNA genome of coliphage Q $\beta$ . As in other viral RNA polymerases (isolated, e.g., from phages MS2 (Fedoroff, 1975), GA (Yonesaki & Haruna, 1981), or SP (Miyake *et al.*, 1971)), only one subunit of the heterotetrameric enzyme is phage encoded (Kamen, 1970; Kondo *et al.*, 1970). This subunit renders replication by the enzyme

specific to its own genome in the context of the host's cellular RNA. The other three components are host encoded nucleic acid binding proteins, namely the ribosomal protein S1 (Wahba *et al.*, 1974) as well as the elongation factors EF Tu and EF Ts (Blumenthal *et al.*, 1972).

The exact roles of the host proteins for replication of viral RNA by Q $\beta$  replicase are yet to be defined. In *Escherichia coli*, EF Tu binds the 3' end of aminoacylated tRNAs to transfer them to the ribosome for protein biosynthesis (Chinali *et al.*, 1974). Hydrolysis of a bound GTP provides the energy necessary for this process to take place. As a subunit in Q $\beta$  replicase, EF Tu seems to be necessary for RNA replication initiation only (Carmichael *et al.*, 1976). Since replication initiates with joining two molecules of GTP complementary to the 3' end of the template, it has been suggested that EF Tu has similar roles both in protein biosynthesis and RNA replication: binding and bringing together GTP and the 3' end of an RNA molecule (Biebricher, 1983, 1987a). However, EF Tu has to be complexed with EF Ts for full functionality in both processes (Blumenthal & Carmichael, 1979).

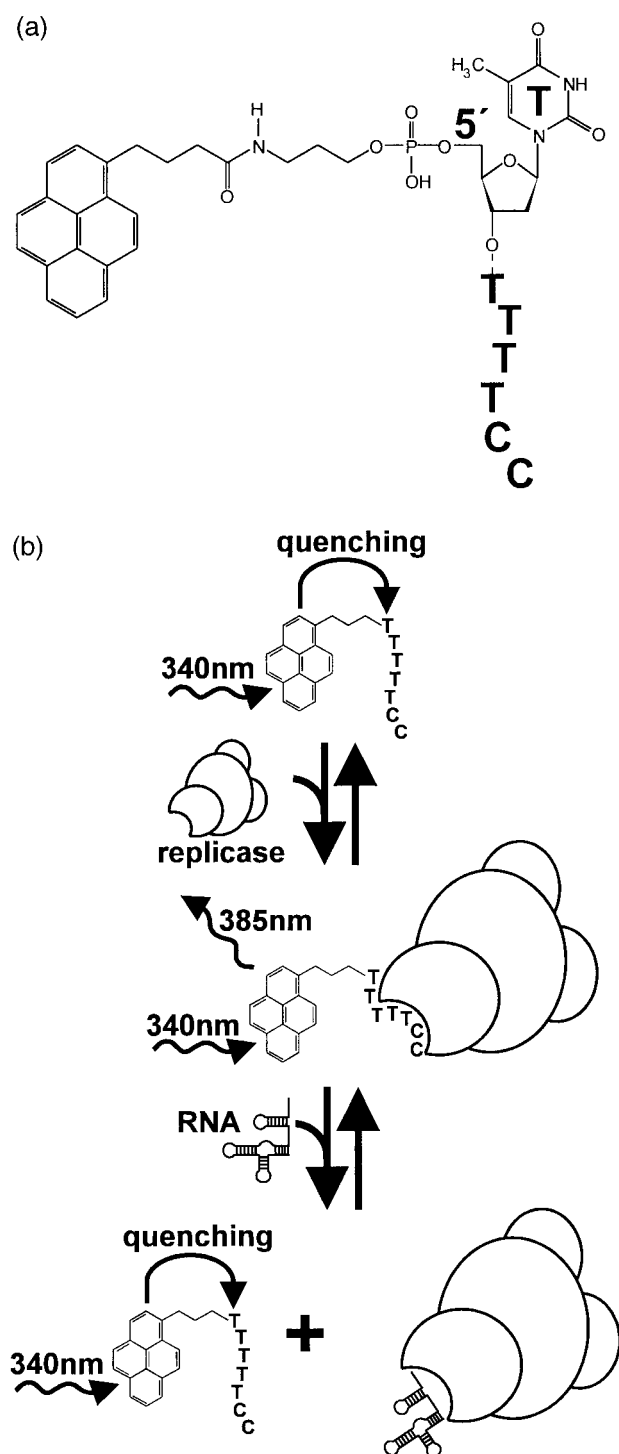
The role of the protein S1 subunit of Q $\beta$  replicase is also obscure. During RNA replication, the plus strand of the viral genome is bound by the polymerase and used as a template for synthesis of the complementary minus strand, and *vice versa*. The strong secondary structure of the single-strands impairs double-strand formation, so that each replicative cycle roughly doubles the previous amount of single-stranded templates. The result is an exponential enrichment of replicated genomic and antigenomic RNAs (Dobkin *et al.*, 1979; Biebricher *et al.*, 1983, 1984). Ribosomal protein S1 is necessary only for the synthesis of the minus strand of the Q $\beta$  genome. Another host factor is also necessary for this step (Spiegelman *et al.*, 1968), that binds the 3' end of the plus strand to enable replication initiation (Barrera *et al.*, 1993). Thus, protein S1 might mediate the interaction of Q $\beta$  replicase with this additional host factor (Biebricher, 1987a). Additionally, S1 has been shown to bind two sites on the plus strand essential for replication, prompting the notion of S1 providing template recognition prior to complementary strand synthesis (Senear & Steitz, 1976; Meyer *et al.*, 1981). In contrast, the minus strand of Q $\beta$ -RNA can be copied *in vitro* using a polymerase preparation lacking both protein S1 and the host factor (Kamen *et al.*, 1972). This observation suggests that minus strand recognition is accomplished by the three other subunits of the Q $\beta$  replicase complex alone.

More recently, a model for the nucleic acid binding properties of the different subunits has been proposed, based on *in vitro* selection of RNA ligands and their UV-crosslinking to Q $\beta$  replicase (Brown & Gold, 1995a, 1996). According to this model, the S1 protein binds a class I of RNA ligands, including the plus strand of Q $\beta$ -RNA, that exhibits a high fraction of unpaired A and C bases.

A second binding site is located on EF Tu and interacts with class II ligands with polypyrimidine stretches. This combination of two independent binding sites would have the benefit of facilitating replication by Q $\beta$  replicase, since the polymerase could stay in contact with both the template and the product strand for a fast reinitiation of synthesis (Brown & Gold, 1996).

Apart from viral RNA (4217 nucleotides long), Q $\beta$  replicase can utilize a number of short-chained (30 to 250 nt) templates for replication. In Q $\beta$  phage infected *Escherichia coli* cells, a variety of RNA variants summed up as "6 S RNA" is generated in conjunction with Q $\beta$ -RNA (Banerjee *et al.*, 1969). Spiegelman (1971) and co-workers selected some efficiently replicated variants in *in vitro* evolution experiments starting from Q $\beta$ -RNA. Numerous midi-, mini-, and nanovariants were generated *in vitro* without the addition of an exogenous template (Kacian *et al.*, 1972; Sumper & Luce, 1975; Mills *et al.*, 1975; Schaffner *et al.*, 1977; Biebricher *et al.*, 1981, 1982; Biebricher, 1987b; Munishkin *et al.*, 1988, 1991; Biebricher & Luce, 1993; Moody *et al.*, 1994). While Q $\beta$ -RNA and the midivariant RNA share a common internal sequence motif that is recognized by the replicase (Nishihara *et al.*, 1983), most replicable RNA variants do not show large sequence similarities. Therefore, considerable efforts have been made to find a general basis for specific RNA recognition by Q $\beta$  replicase. Imaging by electron microscopy (Vollenweider *et al.*, 1976; Barrera *et al.*, 1993), footprinting experiments with RNases (Schaffner *et al.*, 1977; Meyer *et al.*, 1981), gel-retardation and filter retention assays (Werner, 1991), deletion analyses (Schuppli *et al.*, 1994), and *in vitro* selection for RNAs being bound (Brown & Gold, 1995a) or replicated by the enzyme (Zamora *et al.*, 1995; Brown & Gold, 1995b) have been utilized to characterize the RNA-protein interactions in this model system.

We have devised a fluorometric assay employing a 5'-pyrene labeled DNA probe to study interactions of Q $\beta$  replicase with various nucleic acids. By considering the probe sequence and length of the attachment linker, we were able to observe fluorescence quenching through fluorophore-nucleobase interactions in the free probe. Despite its deoxyribose backbone, the probe is readily bound by the RNA-dependent RNA polymerase, as observed through a profound fluorescence increase upon addition of Q $\beta$  replicase. With addition of replicable RNA to the probe-enzyme complex, the fluorescence decreases again. Apparently, the binding site on the enzyme is the same for both DNA probe and RNA, resulting in displacement of the probe from its shielding environment in the complex. This effect was used to access equilibrium dissociation constants of different RNAs and DNAs to Q $\beta$  replicase in a reverse titration assay. The resulting data on affinities of nucleic acid-Q $\beta$  replicase interactions provide insights into sequence specific recognition by this RNA polymerase.



**Figure 1.** Molecular basis of the fluorescence assays. (a) Design of the DNA probe. A hepta-pyrimidine oligodeoxynucleotide was linked *via* a 5'-aminopropyl linker to pyrene butyric acid as the detector fluorophore. (b) Binding of probe and competing nucleic acids to Q $\beta$  replicase. Pyrene (excited at 340 nm) is strongly quenched through interaction with the pyrimidine sequence of the probe, when free in solution. With the probe reversibly bound to the heterotetrameric replicase of phage Q $\beta$ , pyrene becomes dequenched and fluoresces with a spectral peak at 385 nm. If nucleic acid ligands to Q $\beta$  replicase are added, they partially exchange with the bound probe, resulting in a fluorescence decrease.

## Results

### A 5'-pyrene labeled DNA probe is bound and becomes dequenched by Q $\beta$ replicase

Pyrene-labeled DNA or RNA probes previously have been used for studying nucleic acid interactions. For example, the formation of hybrids between complementary RNAs or DNAs could be monitored by exploiting a change in quenching efficiency of the attached pyrene (Koenig *et al.*, 1977; Yamana *et al.*, 1992a,b; Mann *et al.*, 1992; Bevilacqua *et al.*, 1992; Kierzek *et al.*, 1993; Turner *et al.*, 1996; Dapprich *et al.*, 1997). The fluorophore is specifically quenched through (possibly proton-coupled) electron-transfer interactions with proximal pyrimidine residues, that are altered upon base-pair formation (Kierzek *et al.*, 1993; Manoharan *et al.*, 1995; Dapprich *et al.*, 1997).

We have utilized this effect to design a readily available DNA probe in which an attached pyrene moiety is highly quenched (Figure 1(a)). We coupled a pyrene butyric acid moiety *via* an aminopropyl linker to the 5' end of the DNA sequence 5'-d(TTTTTCC), using standard synthesis chemistry (see Materials and Methods). The pyrimidine-rich sequence and the medium length linker favor quenching of the fluorophore by electron transfer from the excited pyrene to the adjacent bases (J.D., N.G.W. & C. Seidel, unpublished results). We found the probe steady-state fluorescence decreased by about 180-fold as compared to uncoupled pyrene butyric acid in aqueous solution (data not shown).

The chosen probe is complementary to an internal loop of the minus strand of MNV-11 RNA (MNV-11(-)), a short and highly structured variant replicated by Q $\beta$  replicase (Figure 5). Surprisingly, addition of this RNA to the probe under various conditions did not result in a change of the observed fluorescence signal (data not shown). Apparently, either the formation of probe-RNA complex does not interfere with pyrene quenching or the complex does not form because of occlusion of the probe's potential binding site through the tight secondary and tertiary structure of the target RNA. However, when Q $\beta$  replicase was added to the probe, the steady-state fluorescence of pyrene increased by two orders of magnitude, and its emission spectrum displayed a more pronounced vibrational peak pattern (Table 1 and Figure 2(a)). Concurrently, the probe showed increased fluorescence anisotropy and an additional long lifetime component of approximately 140 ns (Table 1 and Figure 2(b)). The pyrene moiety obviously becomes strongly shielded from quenching interactions with the attached nucleobases in the presence of enzyme, resulting in the observed dequenching (Figure 1(b)). These effects were not observed with free pyrene butyric acid.

**Table 1.** Fluorescence intensities, anisotropies, and lifetimes of 5'-pyrene-labeled probe 5'-d(TTTTTC), before and after addition of Q $\beta$  replicase and dsMDV-1 RNA

Sample <sup>a</sup>	Relative intensity at 385 [416] nm	Anisotropy <sup>c</sup>	Lifetimes (ns) (rel. amplitudes) <sup>d</sup>
Probe	1 [1]	-0.061	0.73 [0.68], 6.39 [0.32]
Probe + Q $\beta$ replicase	121 [155]	-0.044	5.03 [0.07], 139 [0.93]
Probe + Q $\beta$ replicase + MDV(ds)	— <sup>b</sup>	-0.039	0.30 [0.48], 8.25 [0.09], 142 [0.43]

<sup>a</sup> All measurements were performed in standard buffer (10 mM sodium phosphate, pH 7.0, 100 mM NaCl) at 16°C as described in Materials and Methods. Concentrations were 1.5  $\mu$ M probe and 1.0  $\mu$ M Q $\beta$  replicase for the intensity measurements, 5  $\mu$ M probe for anisotropy and lifetime measurement of the probe alone, and 0.25  $\mu$ M probe, 0.25  $\mu$ M Q $\beta$  replicase, and 1.0  $\mu$ M MDV(ds) for the other anisotropy and lifetime measurements, respectively.

<sup>b</sup> A more detailed description of fluorescence intensities upon reverse titration of probe/Q $\beta$  replicase complex with MDV(ds) is given in Figure 6.

<sup>c</sup> Fluorescence anisotropies were calculated from intensity measurements with polarized light as described in Materials and Methods.

<sup>d</sup> Data sets were fitted as described in Materials and Methods to yield exponential decay components. Their amplitudes were normalized to add up to 1.

### Probe and Q $\beta$ replicase form a stable stoichiometric complex, from which the probe can be displaced by replicable RNA

When an excess of double-stranded MNV-11 RNA (MNV-11(ds)) was added to the mixture of probe with Q $\beta$  replicase (Figure 1(b)), the enhanced pyrene steady-state fluorescence decreased again and its long fluorescence lifetime component was suppressed (Table 1 and Figure 2(b)). MNV-11(ds) is known to bind to a site on the polymerase essential for RNA replication, thereby inhibiting synthesis of the single-strands in late stages of *in vitro* replication (Biebricher *et al.*, 1984; Biebricher 1987a).

Figure 1(b) offers an explanation for the observed reversal of dequenching of the probe-Q $\beta$  replicase complex by addition of minivariant RNA (see Discussion). The pyrimidine-rich DNA probe is assumed to bind reversibly to Q $\beta$  replicase at a site also utilized for binding replicable RNA. Through complex formation, the pyrene fluorophore is shielded from quenching interactions with the probe's pyrimidine residues, resulting in a fluorescence increase. Upon addition of replicable RNA, the probe is displaced from its binding site by competition with the RNA, and it becomes quenched again.

To analyze the probe-Q $\beta$  replicase interaction in a more quantitative way, we performed two titration assays. First, 0.25  $\mu$ M Q $\beta$  replicase were titrated with a stock solution of pyrene-labeled probe. Fluorescence intensity was monitored as a function of probe concentration to extract the equilibrium dissociation constant of the probe-enzyme complex (Figure 3). Second, a reverse titration of probe-Q $\beta$  replicase complex with MNV-11(-) (Figure 5) was performed. The resulting fluorescence decrease was used in a Scatchard plot to infer the stoichiometry of RNA molecules required for displacing the probe from the complex (Figure 4).

Equation (2) (see Materials and Methods) was used to fit the data points from the forward titration of Q $\beta$  replicase with probe in Figure 3 to yield an equilibrium dissociation constant for the

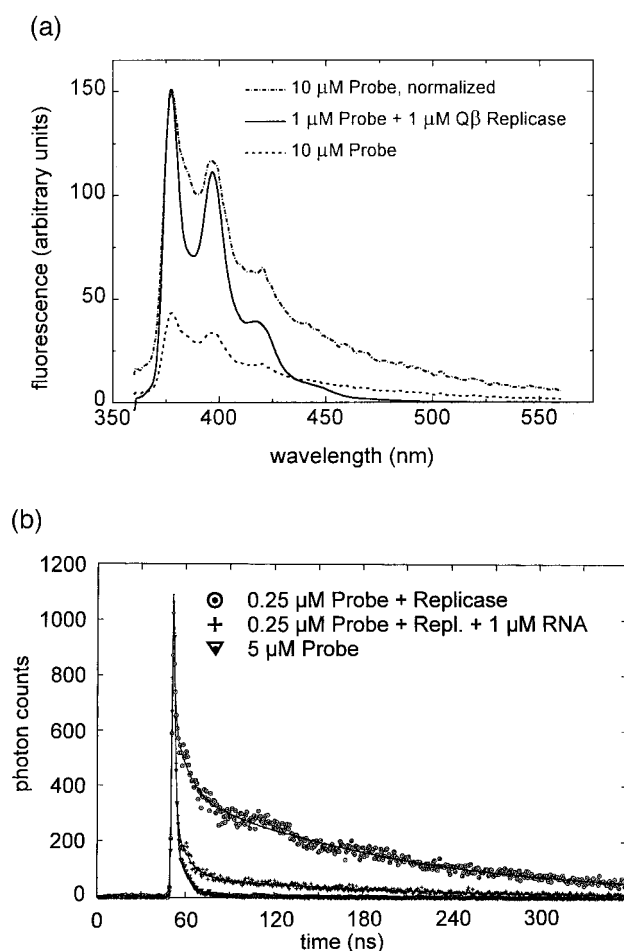
enzyme/probe complex of 11 ( $\pm$ 2) nM (Table 2). This value was reproducible in several titrations monitored either at an emission wavelength of 385 nm or 416 nm. Noteworthy, the fluorescence increase in Figure 3 starts to level off at approximately stoichiometric concentrations of probe and enzyme (0.25  $\mu$ M each). This observation suggests a 1:1 ratio of probe binding to Q $\beta$  replicase.

Analyzing the fluorescence data from reverse titration of probe/Q $\beta$  replicase complex with MNV-11(-) in a Scatchard plot as described in equation (11) (see Materials and Methods) yielded a linear regression line with a *y*-intercept of 0.93 (Figure 4). This figure corresponds to the inverse of the number of binding sites on Q $\beta$  replicase from which MNV-11) is displacing a pyrene-labeled probe. Finding this number to be 0.93 strongly argues for the scheme in Figure 1(b), where one probe molecule becomes bound per Q $\beta$  replicase molecule and can be displaced reversibly by replicable RNA.

### Equilibrium dissociation constants of nucleic acid-Q $\beta$ replicase complexes from reverse titration assays

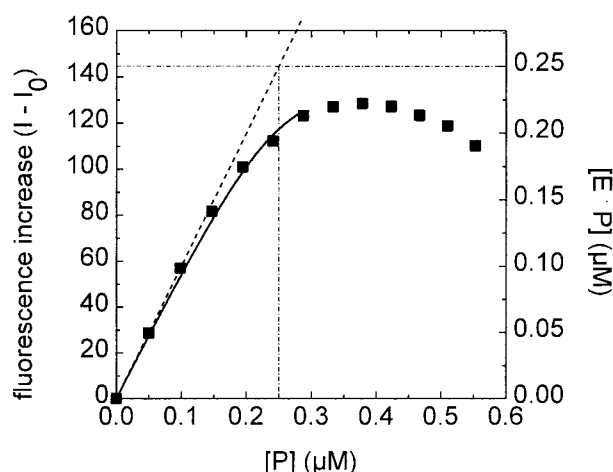
Using the difference in fluorescence of free 5'-pyrene-labeled probe *versus* probe bound to Q $\beta$  replicase, we were able to perform reverse titration assays for a variety of RNAs and DNAs that would displace probe from its complex with Q $\beta$  replicase (Figure 1(b)). The most important of the employed replicable RNAs are depicted in Figure 5. Equation (9) (see Materials and Methods) was used to analyze the fluorescence titration curves by non-linear least-squares regression, as exemplified in Figure 6. The resulting dissociation constants are listed in Table 2.

The dissociation constants ( $K_d$ ) vary from 1.8 nM to 1200 nM, indicating a wide range of affinities between nucleic acids and Q $\beta$  replicase. Alongside replicable RNA, a variety of non-template nucleic acids, such as tRNAs or DNAs, are bound with considerable affinity. The DNA probe binds to the enzyme with an intermediate  $K_d$  of 11 nM. This finding is a prerequisite for accurately measuring



**Figure 2.** Fluorescence properties of the 5'-pyrene-labeled DNA probe, before and after addition of Q $\beta$  replicase and replicable RNA. (a) Steady-state fluorescence emission spectrum of probe in standard buffer (10 mM sodium phosphate, pH 7.0, 100 mM NaCl) at 16°C. After addition of stoichiometric amounts of Q $\beta$  replicase (continuous line), the probe shows enhanced fluorescence as well as a more pronounced peak pattern in comparison to probe alone (broken and dash-dotted lines). (b) Time-resolved fluorescence emission of probe in standard buffer, at 16°C. Without further additives, the probe exhibits a rapid fluorescence decay (triangles). Upon addition of an equal amount of Q $\beta$  replicase, a longer lifetime component appears (circles). Its contribution decreases again with addition of an excess of MDV(ds) RNA (crosses). Fluorescence lifetimes and their amplitudes were derived from the indicated decay curves (continuous lines) as described in Materials and Methods and are detailed in Table 1.

dissociation constants of other nucleic acids in a reverse titration or displacement assay format. If the probe bound too weakly to Q $\beta$  replicase, the reverse titration curves would not change with nucleic acids of different affinities, and a calculated  $K_d$  would not reflect a valid estimate for the dissociation constant. Alternatively, if the probe bound too tightly, it could not be displaced efficiently by other nucleic acids. Thus, the short DNA probe chosen here is not only favorable for ana-

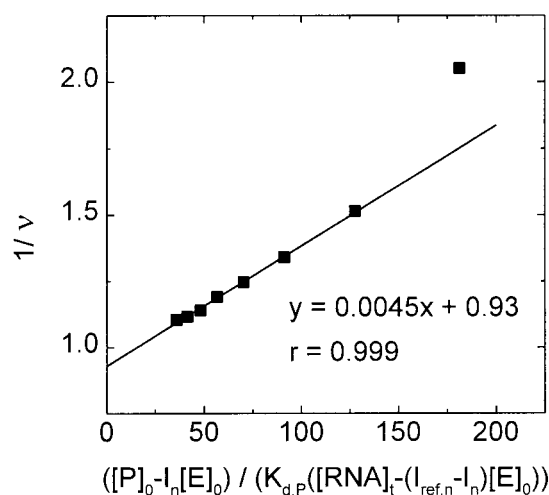


**Figure 3.** Titration of 0.25  $\mu$ M Q $\beta$  replicase with probe in 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, at 16°C. Pyrene fluorescence increase over its initial value  $I_0$  was monitored at 385 nm as a function of added probe concentration. A linear regression line from the initial increase (broken line) was used to correlate the measured fluorescence with the corresponding concentration of enzyme/probe complex [E·P] (dash-dotted lines to scale the right  $y$ -axis; see Materials and Methods). The data points were then fitted with equation (2), as described in Materials and Methods (continuous curve), to yield an equilibrium dissociation constant for the probe/Q $\beta$  replicase complex of  $K_d = 11 (\pm 2)$  nM.

lyses of nucleic acid/Q $\beta$  replicase interactions due to its fluorescence quenching properties, but also because of a well suited dissociation constant.

### Replication of RNA by Q $\beta$ replicase can be monitored in real-time by fluorescence decrease of a pyrene-labeled DNA probe

Recently, Q $\beta$  replicase has been suggested for exponential amplification of reporter RNAs in diagnostic clinical assays (Chu *et al.*, 1986; Kramer & Lizardi, 1989; Burg *et al.*, 1995, 1996; Tyagi *et al.*, 1996; Stone *et al.*, 1996). The observation that a sensitive, easily synthesizable 5'-pyrene-labeled DNA probe can be used to detect binding of replicable RNAs to Q $\beta$  replicase prompts a novel type of fluorescence detection for these assays. We supplemented a standard amplification reaction of MNV-11 by Q $\beta$  replicase with 0.25  $\mu$ M pyrene-labeled probe (see Materials and Methods). Upon addition of the probe, fluorescence increased as observed before, due to probe binding to the polymerase. With exponential amplification of MNV-11 RNA, the probe is increasingly displaced due to competition for the binding site of Q $\beta$  replicase. Consequently, the pyrene fluorescence decreases significantly by about 60% (Figure 7). The time trace of fluorescence thus shows an inverted profile as compared to detection by a free fluorophore such as ethidium bromide or propidium iodide,



**Figure 4.** Scatchard plot of the fluorescence decrease upon titration of probe/Q $\beta$  replicase complex with MNV-11(-) RNA. Equation (2) (see Materials and Methods) was used to calculate the parameters characterizing the interaction of MNV-11(-) with polymerase. The slope of the linear regression line (continuous line, represented by the given equation with a correlation coefficient of  $r = 0.999$ ) yields a dissociation constant of  $K_d = 4.5$  nM, while the  $y$ -intercept defines the number of probe binding sites to  $n = 1/0.93 \approx 1$ .

which exhibits fluorescence enhancement upon intercalation into amplified RNA (Schober *et al.*, 1995; Burg *et al.*, 1995).

## Discussion

Fluorophore-labeled nucleotides and nucleic acids recently have found increasing interest for studying nucleic acid/protein interactions (e.g. Allen *et al.*, 1989; Giedroc *et al.*, 1991; Guest *et al.*, 1991; Delahunty *et al.*, 1994; Carver *et al.*, 1994; Huang & Klingenberg, 1995; Jezewska & Bujalowski, 1996; Thrall *et al.*, 1996). We have devised an assay in which the fluorescence of a 5'-pyrene-labeled DNA probe is enhanced upon 1:1 stoichiometric binding to the RNA-dependent RNA polymerase of coliphage Q $\beta$ . This effect seems to stem from shielding of the fluorophore from quenching effects by the pyrimidine residues of the probe nucleic acid sequence (Manoharan *et al.*, 1995; Dapprich *et al.*, 1997). If nucleic acids are added that compete for the same binding site on Q $\beta$  replicase, the probe fluorescence decreases again.

Upon combining probe and Q $\beta$  replicase, the anisotropy of pyrene fluorescence increases from  $-0.061$  to  $-0.044$  (Table 1). This change is consistent with a probe/enzyme complex forming, that can be expected to result in a slower rotational diffusion and higher anisotropy of the dye on the probe (Lakowicz, 1983). Moreover, a fast ( $< 1$  ns) lifetime component of the free probe is lost with addition of enzyme, while a long ( $\approx 140$  ns) lifetime

**Table 2.** Dissociation constants ( $K_d$ ) of nucleic acid/Q $\beta$  replicase complexes, as derived from forward and reverse titrations of 5'-pyrene-labeled probe/enzyme complex<sup>a</sup>

Nucleic acid	$K_d$ (nM) <sup>b</sup>
Probe 5'-d(TTTTCC)	(11 $\pm$ 2)
MDV(-)	(3.5 $\pm$ 0.7)
MDV(+)	(10 $\pm$ 2)
MDV(ds)	(23 $\pm$ 5)
MNV-11(-)	(4.3 $\pm$ 0.8)
MNV-11(+)	(17 $\pm$ 5)
MNV-11(ds)	(24 $\pm$ 8)
SN0706	(5.1 $\pm$ 0.9)
SN0709	(5.2 $\pm$ 0.9)
DN3	(6.6 $\pm$ 1.2)
Total tRNA ( <i>E. coli</i> )	(38 $\pm$ 7)
Total tRNA (yeast)	(20 $\pm$ 4)
Total tRNA (wheat germ)	(10 $\pm$ 2)
tRNA <sup>Phe</sup> ( <i>E. coli</i> )	(360 $\pm$ 65)
tRNA <sup>Met</sup> ( <i>E. coli</i> )	(31 $\pm$ 6)
tRNA <sup>Val</sup> ( <i>E. coli</i> )	(14 $\pm$ 3)
Synthetic tRNA <sup>ASP</sup> ( <i>E. coli</i> )	(11 $\pm$ 2)
Synthetic tRNA <sup>Ser</sup> ( <i>E. coli</i> )	(10 $\pm$ 2)
dC <sub>20</sub>	(1.8 $\pm$ 1.2)
dT <sub>20</sub>	(2.3 $\pm$ 1.0)
dG <sub>20</sub>	(1000 $\pm$ 250)
dA <sub>20</sub>	(1200 $\pm$ 300)
dC <sub>20</sub> /dG <sub>20</sub> (ds)	(2.9 $\pm$ 0.8)
dT <sub>20</sub> /dA <sub>20</sub> (ds)	(480 $\pm$ 150)

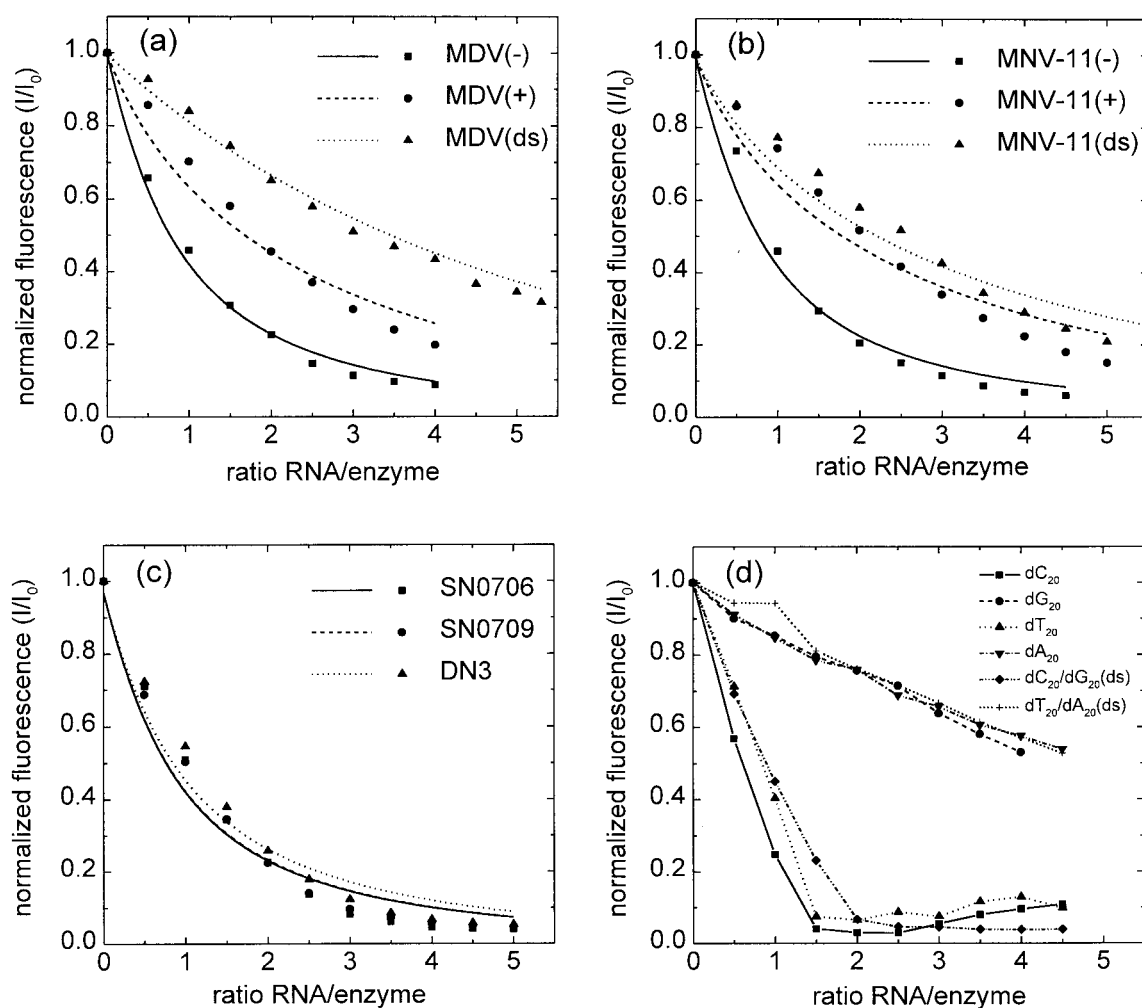
<sup>a</sup> All measurements were performed in standard buffer (10 mM sodium phosphate, pH 7.0, 100 mM NaCl) at 16 °C as described in Materials and Methods. Concentrations for the forward titration were 0.25  $\mu$ M Q $\beta$  replicase in buffer titrated with a 7  $\mu$ M probe stock solution. For the reverse titration, a 17.5  $\mu$ M RNA or DNA stock solution was added gradually to buffer containing 0.25  $\mu$ M Q $\beta$  replicase and 0.38  $\mu$ M probe.

<sup>b</sup>  $K_d$  values were derived from non-linear fitting of titration curves as described in Materials and Methods. The given deviations reflect errors from at least two separate titrations, each analyzed at two emission wavelengths (385 and 416 nm). They also take into account the error from determination of  $K_d$  for the probe/enzyme complex. To obtain valid  $K_d$ s for double-stranded RNA or DNA, it was ensured that the excess of one of the single strands was less than 5% by purification of double-strands on non-denaturing gels.

component appears. Upon addition of a fourfold excess of MDV(ds) RNA, the relative amplitude of the short lifetime component increases again, while that of the long lifetime component decreases (Table 1). Higher concentrations of competing RNA enhance this reversal (data not shown), resulting in the fluorescence decrease upon titration presented here. A reversible exchange of competing nucleic acids on a single site of Q $\beta$  replicase (Figure 1(b)) seems the most likely interpretation of these findings.

The probe fluorescence signal quickly ( $< 5$  minutes) stabilizes after addition of Q $\beta$  replicase, and again after addition of competing unlabeled nucleic acids. Apparently, equilibrium adjustment between free and Q $\beta$  replicase-bound nucleic acids occurs rapidly. This finding is in agreement with the fast association and dissociation rates previously observed for MDV and Q $\beta$  genomic RNAs binding to the polymerase (Werner, 1991). With Q $\beta$  replicase binding a variety of different nucleic acids, including tRNA and DNA, this fast





**Figure 6.** Reverse titration of probe/Q $\beta$  replicase complex with (a) single and double-stranded MDV RNAs, (b) single and double-stranded MNV-11 RNAs, (c) short replicable variants, and (d) homo-oligodeoxynucleotides. For (a) to (c), both the data points for the fluorescence decrease with increasing RNA content as well as the curve fits using equation (9) (see Materials and Methods) are shown. In (d), titration data points are connected for better visibility.

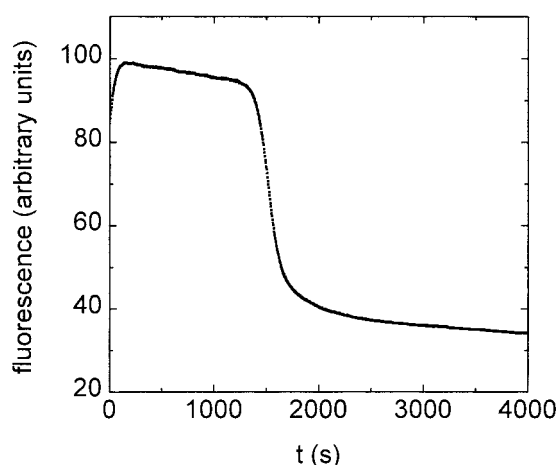
exchange of bound nucleic acids might help the enzyme to screen for Q $\beta$  genomic RNA in the cellular environment of infected *E. coli*. Of the many individual nucleic acid molecules encountering an interaction with the polymerase, only those exhibiting additional properties such as a 5' leader stem and an unpaired C-tract on the 3' terminus will eventually be replicated (Zamora *et al.*, 1995; Brown & Gold, 1995b).

On the basis of the recently proposed model for nucleic acid binding and replication by Q $\beta$  replicase, it seems reasonable to assume that the probe binds to the proposed RNA binding site II, presumably located on subunit EF Tu (Brown & Gold, 1996). This site has been shown to bind pyrimidine tracts of *in vitro* selected RNA ligands and assist in complementary strand initiation (Brown & Gold, 1995a, 1996). Despite its lack of 2'-hydroxyl groups, the hepta-pyrimidine sequence of the probe (5'-d(TTTTTCC)) appears to be sufficient for binding to this site with a nanomolar dissociation con-

stant ( $K_d = 11$  nM). All nucleic acids with pyrimidines employed in the reverse titration assay were able to displace the probe from this site on Q $\beta$  replicase. This observation is consistent with the fact that most replicable RNAs preferably interact with binding site II, except for the Q $\beta$  genomic plus strand (Brown & Gold, 1996).

Since only the binding of one probe molecule per Q $\beta$  replicase was detected by an increase in pyrene fluorescence, and since this molecule most probably binds to RNA binding site II, the equilibrium dissociation constant measured for the probe/enzyme complex also reflects binding to site II only. If more probe molecules bind to Q $\beta$  replicase, e.g. to site I, these additional molecules do not change their fluorescence signal upon binding. The same line of argumentation holds for the reverse titration assays, where the displacement of only one probe molecule per Q $\beta$  replicase was detected by fluorescence quenching (Figure 4). Consequently, the equilibrium dissociation con-





**Figure 7.** Real-time monitoring of RNA amplification by Q $\beta$  replicase. A standard replication reaction of MNV-11 RNA was supplemented with 0.25  $\mu$ M 5'-pyrene labeled DNA probe as described in Materials and Methods. A subsequent increase in amplified RNA concentration resulted in a decrease in fluorescence upon competition of probe with MNV-11 RNA for binding sites on Q $\beta$  replicase.

stants obtained from this competition assay are valid only for site II binding. In contrast to other, less specific techniques for measuring  $K_d$ s of nucleic acid/Q $\beta$  replicase interactions such as gel-retardation or filter binding assays (Werner, 1991; Brown & Gold, 1995a), direct monitoring of binding using the fluorescence assay described here yields information on specific interactions with a single site of Q $\beta$  replicase.

Nanomolar equilibrium dissociation constants for replicable RNAs, as derived from pyrene quenching experiments, generally are consistent with values obtained through gel-retardation or filter binding assays (Werner, 1991; Brown & Gold, 1995a). However, MDV(+) has been described earlier to bind to Q $\beta$  replicase with higher affinity than MDV(-) (Werner, 1991), while in our study they behaved the opposite under fairly similar conditions (Table 2). This discrepancy might relate to the fact that binding RNA to all accessible sites on Q $\beta$  replicase as during gel-shifts or filter retention can only yield an average binding constant for all available sites, such as the suggested sites I and II. As described above, the fluorescence assay can be expected to be more specific for site II binding. MDV(+) has been shown to bind to Q $\beta$  replicase through interactions with nucleotide sequences near the middle of the RNA, that are almost identical to an internal region of Q $\beta$  antigenomic minus strand (Figure 5; Nishihara *et al.*, 1983). Though Q $\beta$ (-) RNA appears to be a site II binder (Brown & Gold, 1996), it cannot be excluded that MDV(+) interacts with other regions as well. Another reason for the observed discrepancy in MDV binding could be that the preparations used here and in the earlier study displayed alternative secondary

structures depending on their thermal history. Indeed, different secondary structures have been proposed for MDV(+) RNA (Nishihara *et al.*, 1983; Chu *et al.*, 1986). The observation that base-pair formation has a large impact on binding affinities of pyrimidines, especially thymine or uracil, could then explain differences in performance of independent RNA preparations.

It has been known for some time that a broad class of replicable RNAs exhibit pyrimidine-rich tracts (Schaffner *et al.*, 1977; Biebricher, 1987b; Munishkin *et al.*, 1988, 1991; Moody *et al.*, 1994). These sequence elements have been proposed to enable binding to Q $\beta$  replicase (Sumper & Luce, 1975) and more recently have been identified as characteristic for site II ligands (Brown & Gold, 1995a). The replicable RNAs employed in our study all contain one or more such C/U-rich domains (Figure 5). The abundance of these elements and their accessibility for the enzyme in the secondary and tertiary structure context of individual RNAs will determine their distinct  $K_d$ s. Discriminative binding affinities for plus and minus strands, as synthesized concomitantly during replication (Biebricher *et al.*, 1983, 1984), might then help in separation of the template from the product strands onto binding sites I and II, or in asymmetric amplification. Interestingly, all three small RNAs derived from early stages of selection experiments (namely SN0706, SN0709, DN3; Figure 5), are binding with comparably high affinities. Efficient binding to Q $\beta$  replicase therefore appears to be an integral feature at this stage, before better replicable variants with additional qualities like MNV-11 evolve.

Total tRNA from *E. coli* binds to Q $\beta$  replicase with lower affinity than tRNA from eukaryotic sources ( $K_d = 38$  nM *versus* 10 to 20 nM, respectively; Table 2). Individual tRNAs that are abundant in *E. coli*, such as tRNA<sup>Phe</sup>, bind with even higher  $K_d$  (360 nM for tRNA<sup>Phe</sup>). In addition, post-transcriptional modifications might play a role in determining binding affinities, since the two tRNAs used as unmodified synthetic transcripts exhibited higher affinities than the natural tRNA utilized in this study (Table 2). Freshly synthesized Q $\beta$  RNA will be unmodified. It seems therefore plausible that discriminative  $K_d$ s may be an adaptation of phage Q $\beta$  to *E. coli* preventing excessive binding to host tRNAs, which do contain a protruding C-rich 3' terminus, one of the prerequisites for binding and replication. Strikingly, tRNA<sup>Asp</sup> and tRNA<sup>Val</sup> show significantly higher binding affinities ( $K_d = 11$  nM in the case of synthetic tRNA<sup>Asp</sup>,  $K_d = 14$  nM with tRNA<sup>Val</sup>), and have both been suggested to act as templates in evolution of replicable RNAs from apparently template-free reactions. It was assumed that they are introduced into these reactions as tightly binding contaminations of Q $\beta$  replicase preparations (Munishkin *et al.*, 1988; Moody *et al.*, 1994).

Double-stranded replicable RNAs such as MDV and MNV bind significantly weaker than their

respective single-strands. However, because of the quasi-species nature of replicated RNAs (Rohde *et al.*, 1995), these preparations most likely represent a mixture of partly mismatched double-strands, still displaying bulged, unpaired bases. It is important to note that a C·G base-pair maintains the binding affinity of an unpaired C base, while a T·A base-pair loses that of a T alone. This finding suggests that Q $\beta$  replicase might display a hydrogen bond acceptor in the major groove of a double-stranded nucleic acid to interact with the exocyclic 4-amino group of cytidine. This interaction would not be possible with the 4-keto group of thymine or uracil, as it requires a hydrogen bond donor instead. For single-stranded nucleic acid binding, additional residues on pyrimidines could be utilized for making contact with Q $\beta$  replicase, such as the 2-keto groups of both C and T or the 3-imino and 3-amino groups of C and T, respectively. A discrimination against purines then might stem from their entirely different stereochemical demands.

In conclusion, the presented method for investigating nucleic acid/Q $\beta$  replicase complexes employing 5'-pyrene-labeled DNA probes yields valuable insights into affinity and specificity of the implied interactions. Moreover, it enables a novel detection method for diagnostic clinical assays based on amplification of reporter RNA by Q $\beta$  replicase. The advantage of the latter is its sensitivity for specific RNA/polymerase interactions as opposed to simple quantification of RNA as with intercalating fluorophores. Finally, because of their simple design and high sensitivity for changes in their molecular environment, 5'-pyrene-labeled oligodeoxynucleotides could be adapted as probes for studying other template/polymerase and, more generally, nucleic acid/protein interactions.

## Materials and Methods

### Materials

Q $\beta$  replicase was isolated from an *Escherichia coli* strain carrying the phage encoded  $\beta$  subunit on a plasmid (obtained from Dr M. A. Billeter, ETH Zürich), essentially as described previously (Sumper & Luce, 1975) with minor modifications (Bauer, 1990). Replicable RNA variants such as MDV-1 (Mills *et al.*, 1973; Kramer & Mills, 1978) or MNV-11 (Biebricher *et al.*, 1981, 1982; Biebricher, 1987b) were amplified from stock solutions by replication in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol, 10% (v/v) glycerol, 0.5 mM each ATP, CTP, GTP, UTP, and 100 nM Q $\beta$  replicase at 37°C. Double and single strands of the RNA variants were prepared from the replication reactions by acrylamide gel electrophoresis in the presence of 1 mM MgCl<sub>2</sub> as described (Mills *et al.*, 1978; Biebricher *et al.*, 1982). SN0706, SN0709 (Zamora *et al.*, 1995), and DN3 (Biebricher & Luce, 1993) were *in vitro* transcribed from plasmids using T7 RNA polymerase. The majority of tRNAs were purchased from Sigma. tRNA<sup>Ser</sup> was *in vitro* transcribed with T7 RNA polymerase from a PCR product encoding the *Escherichia coli* gene with an additional 5' leading sequence of GGCAGCATGTCA. tRNA<sup>Asp</sup> was

a generous gift from C. S. Vörtler and F. Eckstein and had been likewise transcribed from a plasmid encoding the gene from *E. coli*. 20mer homo-oligodeoxynucleotides were synthesized on a Milligene Expedite Synthesizer using standard phosphoramidite chemistry from Glen Research and deprotected according to the manufacturer's instructions. The DNA probe of sequence 5'-d(TTTTCC) was accordingly synthesized on an Applied Biosystems 392 DNA/RNA Synthesizer with a 5'-aminopropyl linker attached. After deprotection and HPLC purification, the probe was coupled with pyrene butyric acid succinimidylester (Molecular Probes, Eugene, OR) according to the manufacturer's instructions, and again purified by reverse phase HPLC to remove uncoupled DNA from the preparation. The concentration of the 5' pyrene-labeled probe was determined using an extinction coefficient of 29,000 at a wavelength of 346 nm, as found for pyrene attached to pyrimidine bases (Kierzek *et al.*, 1993).

### Steady-state fluorescence measurements

Steady-state fluorescence spectra, intensities, and time traces were recorded on a Perkin-Elmer LS5B spectrofluorometer in a thermostated quartz microcuvette (cross section 3 mm  $\times$  3 mm). All measurements were performed in 10 mM sodium phosphate (pH 7.0), 100 mM NaCl as the standard buffer, at a solution temperature of 16( $\pm$ 0.5)°C. Pyrene was excited at 340 nm (band width 5 nm), and fluorescence emission for titration assays was monitored at both 385 nm and 416 nm (band width 10 nm). Photobleaching of the fluorophore under these conditions was found to be negligible.

Forward titration of Q $\beta$  replicase with a 7- $\mu$ M stock solution of pyrene-labeled probe was performed with 0.25  $\mu$ M polymerase in 70  $\mu$ l standard buffer. After each addition of probe, the solution was mixed gently to avoid denaturation of Q $\beta$  replicase. Then the solution was incubated in the cuvette for five minutes to ensure equilibrium adjustment before reading the fluorescence intensity. At this point, the probe fluorescence had become constant. The equilibrium dissociation constant for the complex formation between enzyme (E) and pyrene-labeled probe (P),  $K_{d,P}$ , is then defined as:

$$K_{d,P} = \frac{[E][P]}{[E \cdot P]} = \frac{([E]_0 - [E \cdot P])([P]_t - [E \cdot P])}{[E \cdot P]} \quad (1)$$

with [E], [P], and [E·P] being the concentrations of free enzyme, free probe, and the formed enzyme/probe complex, respectively. These concentrations can be derived from the initial enzyme concentration [E]<sub>0</sub> and the added total probe concentration [P]<sub>t</sub>, as indicated. Equation (1) directly translates into:

$$\frac{[E \cdot P]}{2} = K_{d,P} + [E]_0 + [P]_t - \sqrt{\left(\frac{K_{d,P} + [E]_0 + [P]_t}{2}\right)^2 - [E]_0[P]_t} \quad (2)$$

To infer the enzyme/probe complex concentration [E·P] from the fluorescence measurements, we assumed that during the initial steps of titration (with enzyme still in excess over probe) the probe is virtually quantitatively converted into the complex. Moreover, a fluorescence contribution of probe not bound to enzyme was assumed to be negligible. Thus, a linear regression line for the initial titration steps will link the measured fluorescence to the corresponding concentration of enzyme/probe complex [E·P] (Figure 3). The data points for the

titration could then be fitted with equation (2), varying only parameter  $K_{d,P}$ .

Reverse titrations of the Q $\beta$  replicase-probe complex with different RNAs or DNAs was performed as described above in 70  $\mu$ l standard buffer with 0.25  $\mu$ M polymerase preincubated for 15 minutes with 0.38  $\mu$ M pyrene-labeled probe. In the forward titration, this probe concentration was high enough to saturate the fluorescence signal (Figure 3). Typically, the RNA or DNA stock solutions employed for titration had a concentration of 17.5  $\mu$ M, so that addition of 1  $\mu$ l stock solution would correspond to one equivalent of Q $\beta$  replicase. After gentle mixing and equilibrium adjustment for five minutes, the fluorescence emission was measured. A titration with the same volume of water devoid of nucleic acids was performed in parallel as a measure for signal changes induced just by the mixing process.

The fluorescence of the reference decreased slightly just through titration with water, most probably due to denaturation and adsorptive losses of Q $\beta$  replicase. Its fluorescence value,  $I_{ref}$ , can be expected to correspond to the total enzyme/probe complex concentration,  $[E \cdot P]_v$  that is available for complex formation in any given step of titration. Moreover, during reverse titration with RNA (or DNA), this total enzyme concentration will be distributed between complexes with both probe (E·P) and RNA (E·RNA), so that:

$$[E \cdot RNA] = [E \cdot P]_t - [E \cdot P] \quad (3)$$

In the forward titration of enzyme with probe, the fluorescence increase with enzyme excess is linear and becomes saturated with 0.38  $\mu$ M probe, the concentration utilized in the reverse titrations. The first observation suggests a linear relation between measured fluorescence intensity  $I$  and the enzyme/probe complex concentration (presumably both in the reference and the RNA-titrated sample), while the second argues for full saturation of the enzyme with probe before reverse titration starts. These two assumptions enable us to form equation (3) into:

$$[E \cdot RNA] = \frac{I_{ref}}{I_{ref,0}} \times [E \cdot P]_0 - \frac{I}{I_0} [E \cdot P]_0 = (I_{ref,n} - I_n)[E]_0 \quad (4)$$

with indices 0 referring to initial values (before the first titration step) and n indicating the normalization with the respective initial fluorescence value. With the definition of the dissociation constants for probe,  $K_{d,P}$ , and RNA,  $K_{d,RNA}$ :

$$[E] = K_{d,P} \frac{[E \cdot P]}{[P]}; \quad K_{d,RNA} = \frac{[E] \times [RNA]}{[E \cdot RNA]} \quad (5)$$

one obtains the equation:

$$K_{d,RNA} = K_{d,P} \frac{\frac{[RNA]_t}{(I_{ref}-I)} - [E]_0}{\frac{[P]_0}{I} - [E]_0} \quad (6)$$

where  $[RNA]_t$ ,  $[E]_0$ , and  $[P]_0$  are the total added RNA concentration and the initial enzyme and probe concentrations, respectively. Equation (6) was used to calculate values for  $K_{d,RNA}$  from single data values.

If  $V$  is the initial sample volume in the cuvette,  $x$  the volume of added RNA stock solution of concentration  $[RNA]_{st}$ ,  $n_P$  and  $n_E$  the amounts of probe and Q $\beta$  replicase in the sample, and the normalized fluorescence decrease of the reference,  $I_{ref,n}$ , is represented by a first order decay as in equation (7), then equation (6) can be

formed into equation (8):

$$I_{ref,n} = c \times e^{-\frac{x}{k}} \quad (7)$$

$$I_n^2 - \left( \frac{K_{d,P}[RNA]_{st}}{n_E(K_{d,RNA} - K_{d,P})} x + c \times e^{-\frac{x}{k}} + \frac{K_{d,RNA} \times n_P}{n_E(K_{d,RNA} - K_{d,P})} \right) \times I_n + \frac{K_{d,RNA} \times n_P}{n_E(K_{d,RNA} - K_{d,P})} c \times e^{-\frac{x}{k}} = 0 \quad (8)$$

This second-order polynome yields the solution:

$$I_n = \frac{p}{2} \pm \sqrt{\left(\frac{p}{2}\right)^2 - \frac{K_{d,RNA} \times n_P}{n_E(K_{d,RNA} - K_{d,P})} c \times e^{-\frac{x}{k}}} \quad (9)$$

with

$$p = \frac{K_{d,P}[RNA]_{st}}{n_E(K_{d,RNA} - K_{d,P})} x + c \times e^{-\frac{x}{k}} + \frac{K_{d,RNA} \times n_P}{n_E(K_{d,RNA} - K_{d,P})}$$

The positive sign is valid for:

$$K_{d,RNA} < K_{d,P}$$

the negative one for:

$$K_{d,RNA} > K_{d,P}$$

Equation (9) was used to fit the fluorescence data from reverse titrations with  $K_{d,RNA}$  as the only parameter varied.

For determination of the available probe binding sites on Q $\beta$  replicase, we chose a Scatchard plot. With  $n$  equivalent and independent binding sites, the fraction  $v$  of bound RNA molecules per enzyme molecule is given to:

$$v = \frac{n[E \cdot RNA]}{[E \cdot RNA] + [E \cdot P]} = \frac{n(I_{ref,n} - I_n)}{I_{ref,n}} \quad (10)$$

Using the same definitions as above, equation (10) leads to:

$$\frac{1}{v} = \frac{1}{n} + \frac{K_{d,RNA}}{n} \times \frac{[P]_0 - I_n[E]_0}{K_{d,P}([RNA]_t - (I_{ref,n} - I_n)[E]_0)} \quad (11)$$

By plotting:

$$\frac{1}{v} = \frac{I_{ref,n}}{I_{ref,n} - I_n}$$

against:

$$\frac{[P]_0 - I_n[E]_0}{K_{d,P}([RNA]_t - (I_{ref,n} - I_n)[E]_0)}$$

the slope of a linear regression line will yield the dissociation constant, while the  $y$ -intercept will give the inverse of the number of probe binding sites  $n$ .

All data sets were fitted with the appropriate equation using Marquardt-Levenberg least-squares non-linear regression of the program Origin (MicroCal).

Finally, to monitor RNA synthesis by Q $\beta$  replicase, a standard amplification reaction with 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.1 mM dithiothreitol, 10% glycerol, 0.5 mM each ATP, CTP, GTP, UTP, and 100 nM Q $\beta$  replicase in a 60  $\mu$ l volume was supplemented with 0.25  $\mu$ M pyrene-labeled probe and initiated with 1 pM MNV-11(+) (Figure 5). For efficient replication, a reac-

tion temperature of 37°C was chosen. Fluorescence readouts were automatically recorded every four seconds.

### Lifetime measurements

The fluorescence lifetime of pyrene was measured in a commercial single-photon counting device (Edinburgh Instruments). A nitrogen discharge lamp at 337.2 nm with a flash frequency of 50,000 s<sup>-1</sup> was used for excitation. The time between excitation pulse and emitted single-photon signal was measured by time-amplitude-conversion from a capacitor's potential into a multiple channel analyzer. The signal of a scattering body was used for deconvolution. No significant difference in the fluorescence decay curves could be detected with and without polarizers, proving that decay rates were not significantly influenced by anisotropic effects. All measurements were performed in 60  $\mu$ l standard buffer at 16 °C over at least 20 minutes to ensure statistical significance. The probe and the Q $\beta$  replicase-probe complex were analyzed at concentrations of 5  $\mu$ M and 0.25  $\mu$ M, respectively. 1  $\mu$ M MDV(ds) was added to obtain a signal from a reverse titration-type of experiment. Data sets were fitted by least-squares non-linear regression to yield up to three exponential decay components together with their relative amplitudes.

### Fluorescence anisotropy measurements

Depolarization of fluorescence is predominantly caused by rotational diffusion of the fluorophore and therefore reflects its mobility. The higher the fluorophore mobility is, the more depolarized its emission will be (Lakowicz, 1983). To analyze anisotropies of solutions as a measure for fluorescence polarization, 10 mm Glan-Thompson polarizers were used on an SLM 8000 spectrofluorometer. Fluorescence intensities were measured with the polarizers for excitation (at 340 nm) and emission (at 385 nm) subsequently in all four possible combinations of vertical (v, 0°) or horizontal (h, 90°) alignment,  $I_{vv}$ ,  $I_{vh}$ ,  $I_{hv}$ , and  $I_{hh}$ . Anisotropy  $A$  could then be calculated as described (Lakowicz, 1983) from

$$A = (I_{vv} - g \times I_{vh}) / (I_{vv} + 2g \times I_{vh}), \text{ with } g = I_{hv} / I_{hh}.$$

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