Influenza A virus is an Orthomyxovirus that causes seasonal respiratory infections and occasional severe pandemics and is responsible for substantial morbidity and mortality worldwide. The influenza genome consists of eight single-stranded, negative-sense viral RNA (vRNA) segments encoding 10 major and several auxiliary polypeptides (1–4). The conserved 13 nucleotides at the 5′ end and 12 nucleotides at the 3′ end of each RNA segment display partial and inverted complementarity and form a double-stranded promoter structure created by base-pairing between the two ends (5, 6). Similar promoter structures based on complementarity also may be found at the genomic ends of other single-stranded, negative-sense viruses, such as the arenaviruses and bunyaviruses (7). Comparisons likewise may be drawn with single-stranded, positive-sense viruses, such as flaviviruses, in which polymerase recruitment and binding are guided by circularization of the genome due to interaction between the 5′ and the 3′ untranslated regions (8).

In the influenza virus, the double-stranded RNA promoter is bound by the viral polymerase, which is a versatile RNA-dependent RNA polymerase (RNAP) capable of functioning as a transcriptase, a replicase, and a poly(A) polymerase (reviewed in ref. 9). Three subunits associate to form the polymerase: PB1, the actual polymerase; PB2, shown to have a cap-binding function; and PA, which acts as an endonuclease. Unlike DNA-dependent RNAPs, RNA-dependent RNAPs generally are viral-encoded polymerases, without cellular counterparts, and therefore their interaction with the RNA promoter is a potential target for the development of specific antiviral agents, such as decoy RNAs (10). Despite this, little structural information is available regarding the mechanisms of RNA promoter recognition and binding by the influenza virus RNAP.

During the influenza virus life cycle, the viral RNAP transcribes the vRNA genome into capped and polyadenylated mRNAs using short primers containing a 5′-7-methyl-guanosine cap structure derived from host cell pre-mRNAs. The vRNA segments are also replicated by the RNAP via cRNA intermediates, which in turn are used as templates to make more vRNA (reviewed in ref. 9). To date, it remains unclear how the polymerase commits to either transcription or replication using the same vRNA template. Current models invoke differential binding of RNAP either to the individual terminal ends of the vRNA (or cRNA) or to the dsRNA promoter at different stages of replication and transcription. Although several studies have addressed binding of the influenza virus RNAP to the dsRNA promoter or to the individual terminal sequences (11–18), no quantitative analyses have been performed to measure the affinity (i.e., the dissociation constant Kd) of the trimeric viral polymerase for the individual termini of the vRNA and dsRNA promoter structure. NMR structures of the unbound dsRNA promoter show that it resembles a double-stranded helix, with base-pairing between complementary residues (Fig. L4) (19, 20). Structural information on the vRNA promoter in complex with
the viral RNAP is not available; however, functional studies led to the proposal of a corkscrew model, in which residues 2 and 3 base pair with residues 9 and 8, respectively, on both the 5′ and 3′ ends, forming two tetraloops (Fig. 1B) (21). Supportive evidence for the corkscrew derives from studies showing that the hairpin loops are essential for the endonuclease activity of RNAP (22, 23), that the 5′ strand loop is required for polyadenylation of the viral mRNA (24), and that the polymerase is stabilized through interactions with the promoter in the corkscrew conformation (25). However, the proposed corkscrew structure has never been observed directly.

Here, we have taken advantage of an insect-cell system to obtain highly purified influenza RNAP free from bound vRNA and used it to quantitatively assess binding of the polymerase to single-stranded genomic terminal sequences using fluorescence-based assays. A single-molecule Förster resonance energy transfer (smFRET) assay with alternating-laser excitation (ALEX) (26–28) allowed quantitation of binding to the dsRNA promoter by distinguishing between double-stranded or single-stranded RNA species. The quantitative information obtained provides insight into the molecular mechanisms of influenza transcription and replication. We also extended our assay to measure FRET between fluorescent dyes located at different positions on the dsRNA promoter to map its structure both with and without the polymerase bound; we confirmed that the unbound RNA is a double-stranded helix, and provided direct structural information on conformational changes in the promoter upon protein binding. The distances obtained for the RNAP-bound influenza promoter support a 3D model of the dsRNA in a corkscrew conformation. This work has implications for the mechanisms of promoter binding by single-stranded segmented RNA viruses, as well as the development of inhibitors that target RNAP functions in this important group of pathogens.

Results
Characterizing Interactions Between Influenza RNAP and Single-Stranded RNA. Recombinant RNAP from influenza A/NorthernTerritories/60/68 (H3N2) virus was expressed using the MultiBac system in Sf9 insect cells and affinity purified using IgG-Sepharose followed by size-exclusion chromatography (29). The polymerase was assayed for purity (Fig. S1A) and activity (Fig. S1B) before being used to study binding to short, fluorescently labeled RNAs corresponding to the conserved 5′ and 3′ termini by using an electrophoretic mobility shift assay (Fig. 2A). Polymerase binding to 20 nM wild-type (WT) 5′ vRNA was observed, and was reduced significantly when the mutations G2A, A7U, and C9A were introduced into the RNA sequence (30, 31). The polymerase bound less well to a WT 3′ vRNA, and this binding was reduced by introducing mutations (C2U, U7A, U9A, A11G)

Fig. 1. Two-dimensional models of the vRNA promoter structure. (A) The unbound promoter is largely double-stranded, with base-pairing between the residues that are complementary. The proximal region is defined as residues 1–9, whereas the distal region is defined as residues 11–16 on the 5′ end and residues 10–15 on the 3′ end. (B) The corkscrew model of the vRNA promoter upon polymerase binding. Residues 2 and 3 base pair with residues 9 and 8, respectively, on both the 5′ and 3′ ends, forming two tetraloops.

Fig. 2. Binding of the viral polymerase to single-stranded RNA. (A) Gel-shift assay showing binding of the viral polymerase to vRNA templates labeled with ATTO647N. Twenty nanomolars of RNA was incubated with or without 20 nM polymerase for 15 min at 28 °C before being analyzed by non-denaturing electrophoresis and visualized at $\lambda_{ex} = 640$ nm. Sequences of the RNA templates used are given in the figure; mutations are underlined. (B) Anisotropy curves of the viral polymerase binding to the vRNA templates. Increasing concentrations of polymerase were incubated with 1 nM ATTO647N-labeled RNA for 15 min at 28 °C, and anisotropy was measured in a scanning fluorimeter (Photon Technology International) with $\lambda_{ex} = 640$ nm. Error bars represent the SD from three independent repeats.
and G9C) into the template (30, 31). To measure the affinity of binding accurately, we used a homogenous binding assay based on fluorescence anisotropy, which monitors the degree by which light emitted by a fluorophore on the RNA is polarized (32). Titrating increasing amounts of the polymerase while the WT 5′ end promoter strand was kept at a constant concentration led to an initial increase in anisotropy corresponding to the formation of the RNA–protein complex, until saturation was reached at higher protein concentrations (Fig. 2B). The data were fitted to give a $K_d$ of 2.2 ± 0.6 nM (Materials and Methods). The polymerase showed a lower affinity for the 3′ end of the viral promoter and did not reach saturation within the range of protein concentrations used, indicating a $K_d$ greater than 1 µM. The poor binding exhibited by the WT 3′ vRNA was not a result of the presence of the dye, as we obtained similar behavior for a 3′ RNA with a dye placed further downstream (Fig. S2). Weak binding also was observed during binding studies of the polymerase to the mutated 5′ and 3′ vRNAs. As binding to only the 5′ vRNA reached saturation, we conclude that the RNAP binds the 5′ end of the vRNA with significantly higher affinity than the 3′ end.

The Influenza Polymerase Binds a dsRNA Promoter with High Affinity.

Having characterized the interaction between the RNAP and the individual terminal sequences, we extended our analysis to the double-stranded promoter by using a highly sensitive smFRET assay. Here, FRET was measured between a donor Cy3 dye at position U18 on the 5′ strand and an acceptor ATTO647N dye at position U4 on the 3′ strand of dsRNA molecules diffusing in solution (Fig. 3A). We used ALEX to allow detection of distinct emission signatures for all diffusing species (26–28), and calculated two fluorescence ratios: the uncorrected FRET efficiency (E*), which reports on the donor–acceptor distance, and the ratio S*, which reports on the stoichiometry of donor–acceptor species (Fig. S3). This scheme carries the advantage that doubly labeled dsRNA species can be distinguished from single-stranded donor-only–or acceptor-only-labeled RNAs, thus allowing the specific analysis of polymerase binding to dsRNA rather than to dissociated single-stranded 5′ or 3′ RNA. The FRET efficiency values (E*) obtained for the dsRNA species were depicted as histograms and fitted with Gaussian functions to determine the mean of the distributions (Fig. 3). A WT, doubly labeled promoter gave a single FRET population with a mean E* of 0.57 (Fig. 3B). Titration with increasing amounts of polymerase (2–50 nM) gave rise to a bimodal FRET distribution (Fig. 3 C–G), indicating the presence of two species. The first population was attributed to the unbound RNA (mean E* ∼0.57), and the second population to dsRNA bound to the polymerase (E* ∼0.79). It is unlikely that formation of the higher FRET population is the result of a change in quantum yields of the dyes, because the mean positions of the stoichiometry histograms do not change as a function of increasing polymerase concentration (Fig. S4). The increase in FRET therefore demonstrates that a structural change in the promoter takes place upon polymerase binding, bringing the two fluorophores closer together in space and therefore resulting in increased energy transfer. A similar construct carrying the ATTO647N dye at position 1 on the 3′ end (rather than at position 4; Fig. S5A) showed similar behavior (Fig. S5B), indicating that the fluorescent dyes do not interfere with polymerase binding.

We also tested a polymerase-binding mutant of the dsRNA promoter (containing the mutations discussed in the previous section), labeled at position U18 on the 5′ strand and U4 on the 3′ strand (Fig. 3H). The RNA-only population corresponded to a mean FRET value of E* = 0.59 (Fig. 3I). Addition of either 2 nM (Fig. 3J) or 5 nM RNAP (Fig. 3K) did not result in any change in the FRET distribution, whereas addition of higher concentrations led to formation of only a small amount of the RNA–RNAP complex (E* ∼0.8) (Fig. 3 L–N), suggesting that the affinity of the polymerase for this mutant promoter is much lower than that for the WT promoter.

We note that affinity of the polymerase for the WT dsRNA construct is high, with the complex being clearly detectable even at protein concentrations as low as 2 nM. Using these binding data, we quantified the percentage of bound RNA (relative to the total RNA available) at each protein concentration and fitted these values to determine the dissociation constant. A $K_d$ value of 0.4 nM for the 5′ U18(Cy3)/3′ U4(A647N) promoter was obtained (Fig. 3O); a similar value (0.7 nM) was obtained by using an alternative 5′ U18(Cy3)/3′ U1(A647N) fluorophore labeling scheme (Fig. S5C).

Analysis of Binding to a dsRNA Promoter Using Single-Molecule Quenchable FRET.

The increase in FRET upon polymerase binding demonstrates that the polymerase causes a structural change in the promoter, which moves the two fluorophores closer together. This, however, does not allow us to distinguish between a structural change in the promoter region (residues 1–9) or in the distal promoter region (residues 11–18). To address this, we used quenchable FRET (quFRET), a fluorescence assay that we recently introduced to study promoter opening (33). When two reporting fluorophores are attached close to each other on opposite strands of a dsRNA (a distance of ∼2 nm or less, taking into account the diameter of the RNA helix and the linker lengths of the dyes), they undergo contact-induced quenching, which suppresses fluorescence emission. When the two strands separate (such as in promoter opening), the quenching is lifted, energy transfer between the donor and acceptor is re-established, and a FRET signal is detected. We therefore have used quFRET to study whether the observed structural change brought about by the polymerase binding to the vRNA promoter occurs in the proximal region.

We modified the WT dsRNA promoter so that the Cy3 donor dye on the 5′ end was attached to the residue at position 3, whereas the ATTO647N acceptor dye remained on 3′ U4 (Fig. 4A). No fluorescence was detected using the WT RNA only (Fig. 4B). Upon addition of 2 nM RNAP, a high-FRET population (E* ∼0.84) was observed (Fig. 4C). Addition of higher concentrations of protein increased this population, demonstrating that more complex was formed (Fig. 4 D–G). The loss of quenching between the two fluorophores when polymerase was added suggests opening of the dsRNA in the proximal region of the promoter. A mutant dsRNA promoter (also 5′ U3′(Cy3)/3′ U4(A647N)) (Fig. 4H) was used in a similar experiment; this promoter showed significantly reduced complex formation compared with WT (Fig. 4 M and N). To address the question of whether a structural change occurs in the distal region of the promoter, we also analyzed a WT dsRNA with a donor dye at position 18 on the 5′ end and an acceptor dye at position 13 on the 3′ end (Fig. S6A). The RNA-only population gave a mean FRET value of E* ∼0.82, and addition of 50 nM RNAP did not result in a significant change in the FRET distribution (E* ∼0.83) (Fig. S6B). Taken together, these data demonstrate that the conformational change induced by protein binding to the dsRNA promoter occurs in the proximal promoter region.

Distance Determination and 3D Modeling of the Influenza Promoter.

FRET histograms are commonly depicted in an uncorrected format, as this represents a minimally processed form of the data (refs. 26 and 27 and this paper); however, it previously was shown that FRET data acquired using ALEX can be corrected to determine distances between the reporting fluorophores, which in turn may generate structural information (34). To obtain structural information for the influenza promoter structure in the absence and presence of the polymerase, we converted our...
Fig. 3. Detecting binding of the viral polymerase to a double-stranded vRNA promoter by using smFRET. WT (A–G) and mutant (H–N) dsRNAs were labeled with donor and acceptor fluorophores at positions 4 and 18 and incubated with increasing concentrations of polymerase at 28 °C for 15 min before smFRET spectroscopy combined with ALEX on diffusing molecules was carried out. Ratio $E^*$ represents the uncorrected FRET efficiency, and curves were fitted with Gaussian functions to determine the center of the distributions. Peaks assigned to free WT RNA and free mutant RNA were fixed at mean $E^* = 0.57$ and $E^* = 0.59$, respectively (using values obtained from fitting of the single peaks obtained for the “RNA only” samples), whereas the peaks assigned to the bound fractions were fitted freely. (O) Peaks assigned to bound WT RNA were used to calculate the percentage bound at each concentration, plotted against polymerase concentration, and fitted with a hyperbolic curve to calculate the dissociation constant. Error bars represent the SD from three independent repeats.

Discussion
Polymerase–Promoter Interactions. In this work, we present insights into the mechanisms of promoter binding by the influenza RNAP. No previous studies quantitatively measured the affinity for the dsRNA promoter exist, and we modeled the structures ab initio. We based our models on 2D secondary structures proposed from functional studies for the unbound promoter (17) (Fig. 5A) and corkscrew model (21) (Fig. 5C), which then were translated into 3D space (Materials and Methods). The 3D models for the dsRNA were used as scaffolds for the dyes, and the mean position of each dye was determined using FRET-restrained positioning and screening (FPS) (36, 37).

For the free promoter RNA, the donor–acceptor distances based on the 3D model were ~6.1 nm (5′ U18 and 3′ U1), ~5.2 nm (5′ U18 and 3′ U4), ~3.1 nm (5′ U3 and 3′ U4), and ~3.0 nm (5′ U18 and 3′ U13) (Fig. 5D). These values are consistent with the ones determined experimentally using our corrected FRET efficiencies (Table 1). As a control, we superimposed our 3D structural model with a published NMR structure of the dsRNA promoter (19), and the two were in good agreement (Fig. S7).

Having verified that the values obtained from the 3D model of the unbound promoter RNA closely match our experimentally determined distances, we generated a 3D representation of the polymerase-bound corkscrew promoter structure (Fig. 5D). This was used to measure the distances between the FRET pairs: 5′ U18 and 3′ U1 are located ~4.7 nm apart, whereas 5′ U18 and 3′ U4, 5′ U3 and 3′ U4, and 5′ U18 and 3′ U13 are located ~4.6 nm, ~4.8 nm, and ~3.2 nm apart, respectively (Fig. 5D). These values also are in good agreement with the distances determined experimentally using smFRET (Table 1), strongly supporting the proposal that the promoter is forming a corkscrew structure when bound by the viral polymerase.

Materials and Methods
0.57 and $E^*$ = 0.59, respectively (using values obtained apparent FRET values ($E^*$) into accurate FRET values by correcting for background, cross-talk, and γ-factor effects (the latter accounting for differences in quantum yield and detection efficiency of the fluorophores), and then calculated donor–acceptor distances (Materials and Methods) (reviewed in ref. 35). The distances between the fluorophores located 18 bp (5′ U18 and 3′ U1), 14 bp (5′ U18 and 3′ U4), and 5 bp (5′ U18 and 3′ U13) apart on the free dsRNA were ~6.3, ~5.7, and ~3.7 nm, respectively, whereas the distance between the fluorophores located at positions 5′ U3 and 3′ U4 was presumed to be ~2 nm or less, because no FRET signal could be detected as the result of contact-mediated quenching (Table 1). The distances obtained for the polymerase-bound dsRNA were ~5.2, ~4.6, ~4.6, and ~3.0 nm for the dyes located at positions 5′ U18 and 3′ U1, 5′ U18 and 3′ U4, 5′ U3 and 3′ U4, and 5′ U18 and 3′ U13, respectively (Table 1).

These experimentally determined distances were compared with 3D models of the labeled dsRNA promoter in either the bound or unbound form. Because no 3D representations of the dsRNA promoter exist, we modeled the structures ab initio. We based our models on 2D secondary structures proposed from functional studies for the unbound promoter (17) (Fig. 5A) and corkscrew (21) (Fig. 5C), which then were translated into 3D space (Materials and Methods). The 3D models for the dsRNA were used as scaffolds for the dyes, and the mean position of each dye was determined using FRET-restrained positioning and screening (FPS) (36, 37).

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polymerase resulted in high FRET peaks centered at E* because of quenching of the fluorophores (33), whereas the addition of ALEX on diffusing molecules was carried out. Ratio E* represents the unbound polymerase at 28 °C for 15 min before smFRET spectroscopy combined with and 4 on opposite strands and incubated with increasing concentrations of dsRNAs were labeled with donor and acceptor fluorophores at positions 3 and 4 on the vRNA template. At this stage, the 3′ end of vRNA presumably has to transfer into the active site of PB1, where it interacts with the mRNA primer for transcription initiation. Thus, during initiation, the 5′ and 3′ ends of the promoter separate to allow copying of the 3′ template; however, once the 3′ end has been copied, it likely reassociates with the 5′ end, restoring the dsRNA promoter structure. In fact, it has been proposed that the polymerase association with the 5′ end of the vRNA promoter is the major factor in allowing polyadenylation of viral mRNA (24). Stable association of the polymerase with the 5′ end of the vRNA template while the template is threaded through the active site of the same polymerase inevitably leads to steric constraints. These constraints result in repeated copying of a sequence of five to seven uridine residues close to the 5′ terminus, giving rise to the poly(A) tail of the viral mRNA molecule (reviewed in ref. 9).

Fig. 4. Analysis of binding of viral polymerase to a double-stranded vRNA promoter by using single-molecule quFRET. WT (A–G) and mutant (H–N) dsRNAs were labeled with donor and acceptor fluorophores at positions 3 and 4 on opposite strands and incubated with increasing concentrations of polymerase at 28 °C for 15 min before smFRET spectroscopy combined with ALEX on diffusing molecules was carried out. Ratio E* represents the uncorrected FRET efficiency. No FRET events were detected with RNA only because of quenching of the fluorophores (33), whereas the addition of polymerase resulted in high FRET peaks centered at $E^* = 0.84$.

Current models of influenza virus transcription propose that the RNAP that is bound to the dsRNA promoter within viral ribonucleoprotein (RNP) complexes (introduced into the cell by infecting virions) is responsible for carrying out viral mRNA synthesis (reviewed in ref. 9). The complex of this so-called resident or cis-acting polymerase and dsRNA promoter presumably represents a preinitiation complex. The PB2 subunit of the RNA-bound polymerase binds the 5′-7-methyl-guanosine cap structure of a cellular mRNA (12, 15); subsequently, an endonuclease activity associated with the PA subunit (38, 39) cleaves the cellular mRNA 10–15 nucleotides downstream of the cap. This generates a short capped RNA fragment that serves as a primer to initiate mRNA synthesis on the vRNA template. At this stage, the 3′ end of vRNA presumably has to transfer into the active site of PB1, where it interacts with the mRNA primer for transcription initiation. Thus, during initiation, the 5′ and 3′ ends of the promoter separate to allow copying of the 3′ template; however, once the 3′ end has been copied, it likely reassociates with the 5′ end, restoring the dsRNA promoter structure. In fact, it has been proposed that the polymerase association with the 5′ end of the vRNA promoter is the major factor in allowing polyadenylation of viral mRNA (24). Stable association of the polymerase with the 5′ end of the vRNA template while the template is threaded through the active site of the same polymerase inevitably leads to steric constraints. These constraints result in repeated copying of a sequence of five to seven uridine residues close to the 5′ terminus, giving rise to the poly(A) tail of the viral mRNA molecule (reviewed in ref. 9).

Several models have been proposed for viral replication, during which the negative-sense vRNA is copied into full-length, nonpolyadenylated, positive-sense cRNA which, after assembly into a complementary RNP (cRNP) complex, acts as a template for genomic vRNA synthesis (reviewed in ref. 9). It has been shown that replication of cRNA into vRNA requires additional polymerase molecules (29, 40). According to one model, this additional, “trans-acting” polymerase performs vRNA synthesis, whereas another model proposes that the trans-activating polymerase performs a nonenzymatic role while replication is carried out by the resident polymerase of the cRNP. It is unclear whether replication of vRNA to cRNA also requires an additional, nonresident polymerase. Common to all replication models is the requirement that during the initiation of cRNA synthesis, the 5′ and 3′ ends of the vRNA promoter must separate, and the 5′ end of the vRNA template must be released by the resident polymerase, independent of whether replication is performed by the resident or nonresident polymerase. This ensures that the replicating polymerase does not encounter steric restraints and copies through the sequence of uridine residues located near the 5′ terminus. Although these models are important steps forward in our understanding of viral replication, many questions remain unanswered. For example, the model that proposes that replication is performed by a trans-acting polymerase requires the initial release of the 3′ end by the resident polymerase and subsequent binding by a second polymerase (reviewed in ref. 9). It is difficult to reconcile our data, in which the 3′ end of the vRNA alone has an extremely low affinity for the polymerase, with this idea. Our data are more consistent with a model in which the 3′ end of the template is replicated by the resident polymerase, whereas the additional polymerase would be fulfilling a nonenzymatic role, in agreement with the
trans-activating polymerase model (29). However, it is possible that in vivo other factors also play a role; for example, the interaction of polymerase with the 3′ end of the vRNA may be stabilized by the presence of the initiating nucleotide or small vRNAs that correspond to the 5′ end of each of the vRNA segments and are expressed at high levels in infected cells (41).

Promoter Structure. Functional studies have demonstrated that secondary structure, as well as sequence, of the vRNA promoter is crucial for recognition, binding, and function of the influenza polymerase. The promoter is thought to be a largely base-paired helix in the absence of polymerase (42), whereas several 2D models for the secondary structure of the promoter in the bound state have been proposed, including a duplex (unchanged from the unbound state) (17), fork (31), and corkscrew model (21). Our FRET results are consistent with the unbound dsRNA being a base-paired helix and show that a polymerase-dependent conformational change in the promoter occurs. Using quFRET, we could show that the conformational change occurs in the proximal promoter region (residues 1–9), suggesting that the promoter might be in the corkscrew conformation.

Investigating the FRET changes by using four distinct labeling schemes allowed us to determine the distances between the fluorophores in our assays. The distances obtained characterized an RNA molecule in 3D space; however, no 3D structure is currently available for the influenza promoter in the corkscrew conformation. The unbound promoter was investigated previously using NMR (19); however, this structure (Protein Data Bank ID code 1J07) differs from our construct, as the RNA used was shorter and formed a hairpin. To overcome these limitations, we 3D modeled the RNA in both the bound and unbound conformations. Our experimentally determined distances were in good agreement with those derived from our models and support the base-paired helix structure for the free RNA and the corkscrew structure for the bound RNA. In obtaining molecular distances from accurate FRET measurements, we have made several assumptions, including that the R0 value for our dye pair is not affected by the microenvironment of the fluorophores, that...
the orientational freedom of the dyes is not affected by the presence of the polymerase (hence, we have used a mean dye-orientation factor of 2/3 in all cases), and that our mean FRET efficiencies represent single-state populations rather than fast-interconverting populations with an averaged-out FRET value. It should be noted that all these factors may introduce some uncertainty into our distance assessments, and this may be responsible for the small differences between our modeled distances and those obtained experimentally.

In our experiments using doubly labeled promoter RNA, we obtained a bimodal FRET distribution (Fig. 3). The low-FRET population (E* ≈ 0.57) was attributed to unbound RNA, as it had an E* similar to that obtained from fitting of the single peak of the “RNA-only” sample, whereas the higher-FRET population (E* ≈ 0.79) was attributed to RNA in the bound state. We note, however, that although the high-FRET population saturates with the presence of the polymerase (hence, we have used a mean dye-orientation factor of 2/3 in all cases), and that our mean FRET efficiencies represent single-state populations rather than fast-interconverting populations with an averaged-out FRET value. It should be noted that all these factors may introduce some uncertainty into our distance assessments, and this may be responsible for the small differences between our modeled distances and those obtained experimentally.

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In our experiments using doubly labeled promoter RNA, we obtained a bimodal FRET distribution (Fig. 3). The low-FRET population (E* ≈ 0.57) was attributed to unbound RNA, as it had an E* similar to that obtained from fitting of the single peak of the “RNA-only” sample, whereas the higher-FRET population (E* ≈ 0.79) was attributed to RNA in the bound state. We note, however, that although the high-FRET population saturates with the presence of the polymerase (hence, we have used a mean dye-orientation factor of 2/3 in all cases), and that our mean FRET efficiencies represent single-state populations rather than fast-interconverting populations with an averaged-out FRET value. It should be noted that all these factors may introduce some uncertainty into our distance assessments, and this may be responsible for the small differences between our modeled distances and those obtained experimentally.
FRET efficiencies were converted into molecular distances with the FRET efficiency equation ($E = 1/(1+[\text{R}^2/\text{R}_0^2])$) using a Förster radius ($\text{R}_0$) of 5.9 nm (ATTO-TEC) and assuming a κ = 23.

Three-Dimensional Modeling of the Influenza Virus RNA. Secondary structures for each of the constructs were determined by using the duplexfold module of the RNAstructure web server (49). Secondary structure information was used in the Chimera plugin Assembly2 (50) to construct 3D models. These structures were exported from Assembly2/Chimera and opened in PyMOL. The attachment point for each dye was identified (on the C5′ of the uracil base), and information on the dye characteristics was inserted. The Cy3 dye was characterized by a linker length of 14.2 Å, a linker width of 4.5 Å, and dye radii of 8.2, 3.3, and 2.2 Å (x, y, and z, respectively). The ATTO647N dye was characterized by a linker length of 17.8 Å, a linker width of 4.5 Å, and dye radii of 7.4, 4.8, and 2.6 Å. FPS software was used to calculate the accessible volumes of the dyes and the average dye positions (represented as a sphere) (36, 51). Distances between the average positions of the FRET dye pairs were determined by using the "measure distance" function available in PyMOL.

ACKNOWLEDGMENTS. We thank Mónica Martínez Alonso (University of Oxford) for reagents, and Thorben Cordes (University of Groningen), Timothy Craggs, George Brownlee, and Frank Vreede (University of Oxford) for helpful discussions. This work was supported by European Commission Seventh Framework Program Grant FP7/2007-2013 HEALTH-F4-2008-201418 and Biotechnology and Biological Sciences Research Council Grants BB/J001694/1 and DKKY10/AK (to A.N.K.), by Medical Research Council (MRC) Grants G0700848 and MR/K000241/1 (to E.F.), by an MRC Doctoral Training Award (to A.I.T.), by Wellcome Trust Studentship 092931/Z/10/Z (to N.H.), and by a Junior Research Fellowship from Linacre College, Oxford (to N.C.R.).
Fig. S1. Purified viral RNA polymerase (RNAP) is active in vitro. (A) Coomassie-stained polyacrylamide gel of purified A/NorthernTerritories/60/68 RNAP. Recombinant viral polymerase with a protein-A tag on the PB2 subunit was expressed using the MultiBac system in Sf9 insect cells and affinity purified using IgG-Sepharose followed by size-exclusion chromatography. (B) Transcriptional activity of purified RNAP on viral RNA (vRNA) promoter sequences. Transcription was carried out using double-stranded synthetic RNA templates in the presence of ApG (lane 2) or ApA (lane 3) as a primer (1). Sequences of the RNA templates used are as follows: WT vRNA promoter (lane 2): 5′AGUAGAAACAAGGAGUUU3′ annealed to 5′UUUAAACUCCUUCUUUGCU3′; mutant vRNA promoter (lane 3): 5′AAUAGAUAAGGAGUUU3′ annealed to 5′UUUAAACUCCUUCUUUGCU3′.


Fig. S2. Binding of the viral polymerase to WT 3′ vRNA. Anisotropy curve of the viral polymerase binding to a 3′ vRNA template with the dye at position 18. Increasing concentrations of polymerase were incubated with 1 nM ATTO647N-labeled RNA for 15 min at 28 °C, and anisotropy was measured in a scanning fluorimeter (Photon Technology International) with λex = 640 nm. Error bars represent the SD from three independent repeats.
Fig. S3.  FRET efficiency/stoichiometry (ES) histogram of single-molecule FRET (smFRET) data showing binding of the viral polymerase to a dsRNA promoter. WT dsRNA was labeled with a donor Cy3 dye at position U18 on the 5' strand and an acceptor ATTO647N dye at position U4 on the 3' strand. RNA was incubated with 50 nM of polymerase at 28 °C for 15 min before smFRET spectroscopy combined with alternating-laser excitation (ALEX) on diffusing molecules was carried out. Two fluorescence ratios—the uncorrected FRET efficiency (E*), which reports on the donor–acceptor distance, and the ratio S*, which reports on the stoichiometry of donor–acceptor species—were used to plot an ES histogram. Doubly labeled dsRNA species could be distinguished from single-stranded donor-only or acceptor-only species. Thresholds may be applied to depict the FRET efficiency values (E*) for the dsRNA species as a 2D histogram and allow fitting with Gaussian functions to determine the center of the distributions.
Fig. S4. Stoichiometry histograms as a function of increasing polymerase concentration. WT dsRNA was labeled with donor and acceptor fluorophores at positions 4 and 18 and incubated with increasing concentrations of polymerase at 28 °C for 15 min before smFRET spectroscopy combined with ALEX on diffusing molecules was carried out. Curves representing the ratio $S^*$, which reports on the stoichiometry of donor-acceptor species, were fitted with Gaussian functions to determine the center of the distributions.
Fig. S5. Binding of the viral polymerase to a double-stranded vRNA promoter labeled at positions 1 and 18. (A) WT dsRNA was labeled with donor and acceptor fluorophores at positions 1 and 18. (B) RNA was incubated with increasing concentrations of polymerase at 28 °C for 15 min before smFRET spectroscopy combined with ALEX on diffusing molecules was carried out. Ratio $E^*$ represents the uncorrected FRET efficiency, and curves were fitted with Gaussian functions to determine the center of the distributions. Peaks assigned to RNA only were fixed at mean $E^* = 0.49$, whereas the peaks assigned to the bound fractions were fitted freely. (C) The percentage bound of the RNA at each concentration was calculated, plotted against polymerase concentration, and fitted with a hyperbolic curve. Error bars represent the SD from three independent repeats.
Fig. S6. Binding of the viral polymerase to a double-stranded vRNA promoter labeled at positions 13 and 18. (A) WT dsRNA was labeled with acceptor and donor fluorophores at positions 13 and 18, respectively. (B) dsRNA alone (Upper) or after incubation with 50 nM of polymerase at 28 °C for 15 min (Lower) was analyzed by smFRET spectroscopy combined with ALEX. Ratio \( E^* \) represents the uncorrected FRET efficiency, and curves were fitted with Gaussian functions to determine the center of the distributions.

Fig. S7. Comparison of the 3D model of free promoter RNA with an NMR structure. Our 3D modeled structure of the free promoter RNA (3′ strand shown in blue; 5′ strand shown in brown) was superimposed on a previously described NMR structure (shown in yellow) (Protein Data Bank ID code 1JO7) (1). There is good homology between the two, with the exception of the distal end, where the NMR structure is shorter than the modeled RNA template.

<table>
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<tr>
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<th>AA Bkg., cps</th>
<th>DA Bkg., cps</th>
<th>$E_0$</th>
<th>Donor leakage</th>
<th>$S^*$</th>
<th>$S_0$</th>
<th>Direct acceptor excitation</th>
<th>$\gamma$</th>
<th>$E$</th>
<th>Distance, nm</th>
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AA, acceptor excitation, acceptor emission; Bkg, background; DA, donor excitation, acceptor emission; DD, donor excitation, donor emission; $E_0$, mean of donor-only population; $S_0$, mean of acceptor-only population; $\gamma$, gamma factor.
### Table S2. Correction factors used during accurate FRET calculations (RNA + RNAP)

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<th>AA Bkg, cps</th>
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AA, acceptor excitation, acceptor emission; Bkg, background; DA, donor excitation, acceptor emission; DD, donor excitation, donor emission; E0, mean of donor-only population; n/a, not applicable; S0, mean of acceptor-only population; γ, gamma factor.