Interaction of influenza virus polymerase with viral RNA in the ‘corkscrew’ conformation

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The influenza virus RNA (vRNA) promoter structure is known to consist of the 5′- and 3′-terminal sequences of the RNA, within very narrow boundaries of 16 and 15 nucleotides, respectively. A complete set of single nucleotide substitutions led to the previously proposed model of a binary hooked or ‘corkscrew’ conformation for the vRNA promoter when it interacts with the viral polymerase. This functional structure is confirmed here with a complete set of complementary double substitutions, of both the regular A:U and G:C type and also the G:U type of base-pair exchanges. The proposed structure consists of a six base-pair RNA rod in the distal element in conjunction with two stem–loop structures of two short-range base-pairs (positions 2–9; 3–8). These support an exposed tetranucleotide loop within each branch of the proximal element, in an overall oblique organization due to a central unpaired A residue at position 10 in the 5′ sequence. Long-range base-pairing between the entire 5′ and 3′ branches, as required for an unmodified ‘panhandle’ model, has been excluded for the proximal element, while it is known to represent the mode of interaction within the distal element. A large number of short-range base-pair exchanges in the proximal element constitute promoter-up mutations, which show activities several times above that of the wild-type in reporter gene assays. The unique overall conformation and rather few invariant nucleotides appear to be the core elements in vRNA recognition by polymerase and also in viral ribonucleoprotein packaging, to allow discrimination against the background of other RNA molecules in the cell.

Introduction

The genome of influenza A virus consists of eight different single-stranded viral RNA (vRNA) molecules of negative polarity. While 13 and 12 nucleotides, respectively, are identical at the 5′ and 3′ ends among all vRNA segments, these terminal sequences are also largely, but not perfectly, complementary to each other, extending out to 16 and 15 nucleotides each on average. The three additional base-pairs specific for the various vRNA segments are also mostly conserved for any particular type throughout all individual virus isolates.

The 5′- and 3′-terminal sequences of the vRNA molecules together constitute the promoter structure, in a stepwise interaction with viral RNA polymerase (Li et al., 1998). Following an initial electron microscopic observation that demonstrated that both ends of a vRNA molecule together are involved in polymerase binding, this interaction of the two termini has been described as an RNA ‘panhandle’ structure (Hsu et al., 1987). In several of the early in vitro and, in part, in vivo analyses, this original structural interpretation was supported (Li & Palese, 1992; Piccone et al., 1993; Baudin et al., 1994), but the results of other studies contradicted this interpretation in regard to the extent of base-pairing between the 5′ and 3′ vRNA ends (‘fork structure’; Fodor et al., 1995). Binding of viral polymerase to the two vRNA termini was specifically confirmed in further reports (Yamanaka et al., 1991; Tiley et al., 1994).

A detailed analysis of the promoter structure and function by site-directed mutagenesis was made possible by the development of reverse-genetic systems applicable to recombinant influenza virus vRNAs, employing either in vitro transcription of cDNA constructs by T7 RNA polymerase followed by in vitro reconstitution of ribonucleoproteins
reporter genes such as chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP). Plasmids pHLL2024 and pHLL1844, which were used as reference constructs in the murine and human promoter series, respectively, carried an influenza virus RNA promoter-up variant consisting of three point mutations in the 3' promoter sequence (5'-G3A, U5C, C6U; nucleotide positions marked in this way refer to residues counted from the 3' end) as originally designed for plasmid pHLL1104 (Neumann & Hobom, 1995). The non-coding regions beyond the promoter elements were derived from segment 4 (in the murine promoter series) or from segment 5 (in the human promoter series), which in direct comparisons, i.e. connected to identical promoter variants, did not differ (E. Hoffmann & G. Hobom, unpublished).

Most of the variations in the 5' and 3' vRNA promoter regions were constructed by using pHLL1261 as a basic construct (R. Flick & G. Hobom, unpublished). After restriction by BsmBI, the plasmid can incorporate in the equivalent position and in an orientation-specific manner BsmBI-restricted PCR fragments that extend across the entire CAT (or GFP) gene, while the promoter variants, as designed for a particular construct, are carried in the flanking PCR primer sequences. The central segment of the inserted PCR fragment was either determined fully by sequencing, or sequenced across the flanking regions, and exchanged for authentic, i.e. non-PCR produced, DNA by using internal unique restriction sites.

**Cells and viruses.** Influenza A/FPV/Bratislava (H7/N7/1979) virus was propagated in MDCK cells and all passaging of virus supernatants containing recombinant influenza viruses was done on sub-confluent plates of MDCK cells. For DNA transfection and consecutive FPV helper-virus infections, we used either murine B82 cells (a mouse L cell line), in the case of plasmids containing the murine RNA polymerase I promoter, or 293-T cells, in the case of plasmids designed for vRNA expression by the human RNA polymerase I system.

**Lipofectamine DNA transfection and influenza helper-virus infection.** For DNA transfection, approximately 10^7 sub-confluent B82 or 293-T cells were used. Five µg of the respective plasmid DNA or of pAM500, a P_FPV-GFP plasmid used for determination of transfection yields, plus 30 µl Lipofectamine (1 µg/µl; Gibco-BRL) were mixed in serum-free Dulbecco’s minimal essential medium (DMEM) and incubated for 15 min at room temperature. The cells were washed twice with serum-free medium and incubated with the DNA–Lipofectamine mixture for 5 h. After further incubation with DMEM containing 10% foetal calf serum (FCS) for 15 h, the transfected cells were washed with PBS supplemented with 2.5 mM MgCl₂ and 3.4 mM CaCl₂ and superinfected with influenza helper virus (influenza A/FPV/Bratislava virus) at an m.o.i. of 1–3. After 30–45 min, the cells were washed again and incubated with DMEM plus 10% FCS for 8–10 h. Transfection yields in this procedure of 12–15% for B82 and 50–60% for 293-T cells, as observed in parallel GFP transfection, were accepted for further treatment.

**Serial passage of virus-containing supernatants.** At 8 h post-infection, DNA-transfected and helper-infected B82 or 293-T cells were harvested for CAT assays and the corresponding supernatants were used for passaging of virus progeny. Cell debris was removed by centrifugation (1800 g; 5 min) and 1 ml supernatant was transferred to approximately 10^7 MDCK cells and incubated for 30–45 min. After transfer to FCS-containing medium and further incubation for 8 h, CPE was observed in the controls and cells and supernatants were treated or passaged in a next round as before.

**CAT assay.** Cell extracts were prepared as described by Gorman et al. (1982). In a first round, 50 µl of undiluted, 1:10- and 1:100-diluted cell lysate was routinely mixed with 10 µl acetyl CoA (4 mM) and 7.5 µl fluorescent-labelled chloramphenicol (boron dipyrromethane difluoride fluorophore: BODIPY CAM substrate; ‘Flash Cat’ kit; Stratagene) and incubated at 37 °C for 3 h. According to initial levels, additional selected

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**Methods**

**Plasmid construction.** Plasmids designed for expression of influenza virus RNA molecules by RNA polymerase I in vitro carried either the murine or human rDNA core promoter region (−251 to −1 or −411 to −1 relative to the RNA precursor 5'-end, respectively). In both cases, the tDNA terminator sequence was derived from murine rDNA (Zobel et al., 1993). Between these two external tDNA elements, influenza virus cDNA constructs were interspersed exactly in the antisense orientation, with influenza virus coding sequences replaced by reporter genes such as chloramphenicol acetyltransferase (CAT) or green fluorescent protein (GFP). Plasmids pHLL2024 and pHLL1844, which were used as reference constructs in the murine and human promoter series, respectively, carried an influenza virus RNA promoter-up variant consisting of three point mutations in the 3’ promoter sequence (5’-G3A, U5C, C6U; nucleotide positions marked in this way refer to residues counted from the 3’ end) as originally designed for plasmid pHLL1104 (Neumann & Hobom, 1995). The non-coding regions beyond the promoter elements were derived from segment 4 (in the murine promoter series) or from segment 5 (in the human promoter series), which in direct comparisons, i.e. connected to identical promoter variants, did not differ (E. Hoffmann & G. Hobom, unpublished).

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concentrations of reference and specific lysates were used in a second round for a semi-quantitative determination of CAT activities relative to the reference construct (equal to 100%) within the limits of 10–40% substrate consumption. An example taken from a serially diluted assay is shown in Fig. 1(D). For extraction of reaction products, 0.5 ml ethylacetate was added and, after centrifugation for 1 min at 15 000 g, the upper phase containing the chloramphenicol substrate and product molecules was isolated and the solvent was evaporated. The pellet was resuspended in 20 µl ethylacetate and the acetylchloramphenicol reaction products were isolated from substrate chloramphenicol by TLC (plates; 20 x 20 cm, silica gel 60) with a mobile phase of chloroform–methanol (87:13). Finally, the reaction products were visualized by UV illumination, documented by photography and evaluated with the WinCam program (Cybertech Berlin). Except for Fig. 1(D), the CAT activities shown are taken from 50 µl cell lysate reactions to allow a direct comparison with inactive or nearly inactive promoter variants in the same series. The relative activities have been calculated on the basis of correlated CAT activities obtained from diluted cell lysates. Relative activities tabulated in Figs 1 and 2 represent mean results from three or more independent experiments.

CAT assay data presented in the upper parts of Figs 1 and 2 were not taken from DNA-transfected and helper-infected (primary) B82 cells, but from infected (secondary) MDCK cell lysates after passage of the recombinant virus-containing supernatants. In infection, helper and recombinant vRNA segments are transcribed and amplified in parallel with the same kinetics, as opposed to the situation in the original DNA-transfected cell, which is infected by helper virus 15 h later. Activities obtained in MDCK were (i) greater, due to another round of differential amplification, and (ii) showed less variation in comparison with CAT assays performed for B82 cells.

Results

Complementary double exchanges in the base-paired promoter regions

The influenza virus promoter structure, which consists of both termini of a vRNA molecule, may be separated into three parts: a proximal element (the first nine nucleotides from both ends of the molecule), followed by angular nucleotide, A10, which has no counterpart in the 3’ sequence, and the distal promoter element, consisting on average of six RNA base-pairs, i.e. positions 11–16 at the 5’ end, which in most vRNAs are exactly complementary to positions T0 to T5 at the 3’ end. Despite the overall sequence conservation, it has been shown repeatedly that compensatory double exchanges in the distal element, i.e. reconstitution by another complementary RNA sequence, will regain the full level of promoter activity, while single nucleotide exchanges disrupting the structure abolish expression of an adjacent reporter gene (Tiley et al., 1994; Fodor et al., 1995; Flick et al., 1996). The same structural elements are also present in other orthomyxovirus RNAs (Stoeckle et al., 1987; Lee & Seong, 1996).

It is well known that RNA double helices are not recognized by interacting proteins in a sequence-specific manner, but only as structural units (Wyatt & Tinoco, 1993). In the promoter-distal element, even the length is not recognized specifically. Confirming the results of an in vitro study (Luo et al., 1991), we observed in our analysis in vivo that, in addition to the naturally observed length variation between five and seven base-pairs, deletion down to four or even three base-pairs led to a severe reduction, but not to a complete loss, of function. Similarly, only partial inactivation was observed in a stepwise extension beyond seven base-pairs in the distal element of the influenza A virus promoter structure (not shown), while nine base-pairs are generally observed in wild-type influenza B virus (Lee & Seong, 1996).

While these data support the requirement for a double-stranded structure in the promoter-distal element at some stage during the propagation cycle, several experiments that introduced either an A:C mismatch or a G:U RNA base-pair into either of the central positions of the short RNA double strand resulted in inactive versus nearly fully active promoter variants. These data suggest that the double-stranded distal element is required as part of the vRNA promoter structure, since the opposite result would have been expected for an element active in the cRNA promoter structure during influenza virus propagation (Flick et al., 1996).

Starting with a full set of 53 single substitution variants in the proximal promoter element and applying the same criteria of complementary double exchanges (in two steps) as developed for the distal element, we proposed previously a binary hooked or ‘corkscrew’ conformation for the overall vRNA promoter structure in the recognition stage. Most significantly, the model includes short-range base-pairing in the proximal element, i.e. between positions 2 and 3 versus 9 and 8, at both ends of the molecule (Flick et al., 1996). This conclusion was based at that time on a rather limited set of complementary double exchanges, complete in every possible variation only for positions 3 and 8 at the 3’ end, plus the proven inability to demonstrate long-range base-pairing in the same region according to the ‘panhandle’ model.

In extending that original result to all four short-range base-pairs, as predicted for the 5’ and the 3’ hook, in covering every potential alternative base-pair at these co-ordinate, complementary positions, we demonstrate that this larger set of data supports the original conclusion (Fig. 1). With two exceptions, every base-pair variation is allowed in any of the four short-range base-pairing positions predicted by this model, and yielded a functional promoter even if at widely different levels of activity. In line with this conclusion, all of the single-step transversion variants within any of these base-pairs were rendered inactive (see two examples in Fig. 1C). Again, the vRNA wild-type promoter sequence is particularly low in its activity (represented by pHLS2428; 3–8: G–C) and its activity is surpassed by almost any other base-pair variant. While the complete set of data has been obtained in the murine RNA polymerase I expression system, a large number of the variants were also tested by using human RNA polymerase I expression in 293-T cells, with very similar results (not shown).

Among the full set of base-pair variants, as represented in Fig. 1(B), we observed a construct (3–8: A–U; pHLS1920) that
A short-range base-pair substitutions in the proximal vRNA promoter element. (A) The vRNA 'corkscrew' conformation is drawn as a model against a schematic representation of its activating interaction with the tripartite viral polymerase. In addition to three invariant positions in the exposed tetranucleotide loops, the angular residue A10 and the (separate) polyadenylation element U5–6 are indicated. Thin lines between the 5′ and 3′ vRNA nucleotide chains refer to base-pairs identified in the proximal and distal promoter elements. (B) Tabulation of mean CAT activities for all single base-pair variations in either of the four short-range base-pairs (obtained via dilution series) relative to reference construct pHL2024 (100%). Plasmid pHL2428 represents the wild-type promoter sequence (3–8: G:C). (C) Examples of CAT assays with MDCK cell lysates obtained at 8 h post-infection by recombinant influenza viruses. Supernatants isolated at 8 h after DNA transfection plus helper-virus infection of B82 cells were used for infection of MDCK cells. The series shown concerns base-pair 3–8, as indicated above the lanes. (D) Comparative CAT assays for pHL1920 versus pHL2024 after a hundredfold dilution relative to (C), an example taken from an individual dilution series, as used to measure relative CAT activities.

was clearly superior to all previously determined promoter-up mutants in its level of activity. For a more complete demonstration of promoter activities in this case, additional CAT assay data, as taken from the step-wise dilution series used in the relative measurement of pHL1920 versus pHL2024 (formerly pHL1104, inserted at that time into a different plasmid), are shown in Fig. 1(D) in a 1:100 dilution relative to Fig. 1(C) (see Methods).

Only two of the base-pair variants involving both 5′ positions 2 and 9 were inactive in this comparative promoter analysis. As a possible explanation, we point out that only in these two cases does the sequence variation introduced result in an uninterrupted stretch of 11 purine residues (positions 4–14), which might interfere with formation of a 5′ hooked base-pair structure because of stacking interactions. The opposite effect may contribute to the increase in activity of pHL1920, which because of the base-pair exchange at positions 3 and 8 results in the interruption of that series with two pyrimidines instead of only one. Variations in activity likely to be due to interference by stacking interactions were also observed in other cases, e.g. in comparing pHL1948 and pHL2428 (wild-type), in which a similar effect may be seen on the complementary strand.

The full set of base-pair variants in the promoter-proximal element was also prepared for an otherwise identical series of constructs carrying a (wild-type) 5′U instead of a (mutant) 5′C residue, with essentially the same results (not shown). Again, almost all base-pair variants yielded active promoters, in essentially every case somewhat below or at most in the same range of activity as in Fig. 1, with the same two base-pair 2–9 mutants remaining inactive. Of the three original substitutions proposed as constituting the full-level promoter-up variation (in pHL1104; Neumann & Hobom, 1995), mutation U5C appears therefore to be of minor importance and the overall effect is due mainly to G3A and C8U; i.e. a single complementary base-pair variation in the 3′ branch with regard to the proposed secondary structure.

In a similar additional round of analysis of the distal double-stranded RNA element, all four base-pair positions identified in the proximal element were converted by transition into both G:U and A:C pairs of RNA nucleotides. In every case in both the 5′ and 3′ branches, the activity observed for G:U RNA base-pairing either came close to the full level of activity of a G:C base-pair in the respective position, or was at least superior in activity to the corresponding A:C pair (Fig. 2). The same bias was also observed in the original DNA-transfected cell, early as well as late after helper-virus infection. From these observations, we conclude that the data obtained in this in vitro analysis, even though it generally covered a complete propagation cycle, primarily reflect the properties of the 5′ plus 3′ vRNA promoter structure. This is in line with an approximately 10:1 ratio of vRNA to cRNA molecules in cell lysates (Mukaigawa et al., 1991). While in a recent publication (Pritlove et al., 1999), the 5′ branch of the 'corkscrew' structure
has also been observed in an experiment in vitro, these authors failed to confirm the secondary structure of the 3' branch. This appears to be due to their use of a rather high concentration of ApG primer dinucleotide, which is likely to interfere with 3' secondary structure formation during initial recognition of vRNA by polymerase.

Since all of the four ‘corkscrew’ base-pairs in the proximal structural element may be exchanged for other pairs of residues, polymerase cannot recognize these in a nucleotide-specific way. Instead, these base-pairs might be recognized structurally, e.g. because of their major stabilizing contribution in supporting and exposing the intervening tetranucleotide loops (positions 4–7) in a correct position and orientation. This appears to be important specifically for those nucleotides in the loop, such as G5, that are found to be invariant and are likely to be recognized directly by viral polymerase (Flick et al., 1996). No variation in size of either tetranucleotide loop sequence was possible by insertion or deletion of single nucleotides.

Promoter ‘corkscrew’ versus ‘panhandle’ structure

In another series of plasmid constructs, the promoter sequence was changed in such a way to reduce maximally the number of potential long-range (‘panhandle’) base-pairs formed between the complementary 5' and 3' vRNA sequences. Only those nucleotides that have either been determined to be invariant or are required for short-range base-pairing according to the ‘corkscrew’ model were left unchanged. In order to achieve this result, residues 4 to 6 in the 3’ tetranucleotide loop sequence, which are known to be variable individually with no reduction in promoter activity (Flick et al., 1996), were altered simultaneously into a sequence that was no longer complementary to positions 4 to 6 of the 5’ sequence. This procedure could not be extended to position 7, since both A7 and U7 constitute invariant nucleotides, i.e. at some stage of interaction they are recognized individually. The resulting triple-variant promoter construct, pHLL2664, retained 91% activity relative to the reference, pHLL2024 (Fig. 3).

In the next step of this series, an additional complementary double exchange was introduced into the promoter structure at positions 3 and 8 of the 5’ sequence. This U:A to A:U base-pair transversion is equivalent to pHLL1920 versus pHLL2024 in Fig. 1, except for the differences introduced before in the proposed 3’ loop sequence. In the resulting construct, pHLL2626, the number of potential long-range base-pairs in the proximal element was reduced further to a nominal four (1–7; 2–8; 7–9; 9–15). These are isolated from each other, however, and are therefore unlikely to exist and to support collectively any overall long-range base-pairing; all four base-pairs required for short-range interactions are maintained in pHLL2626, however (compare individual 3:8 structures in Fig. 3 A and B). The resulting promoter construct maintained a level of 82% activity, as shown in Fig. 3 (C).

In the final step of this series, another single substitution was introduced into the promoter structure at position 8 (U8A; pHLL2644), which at the same time both disrupted the short-range base-pair 3–8 (A:A) and also reinstated an additional long-range base-pair, 8–15 (A:U). Furthermore, this additional base-pair would be expected to close the gap between the pre-existing but isolated long-range base-pairs 7–9 and 9–15 (located adjacent to the fully base-paired distal promoter element, 11–16; 10–15). Altogether, the construction of pHLL2644 and of the entire series in Fig. 3 was designed to maximize the potential influence of the nucleotide substitution U8A on short-range or ‘corkscrew’ versus long-range or ‘panhandle’ base-pairing through the simultaneous destruction of one of the four short-range base-pairs and the effective reinstatement of three long-range base-pairs. The experimental result indicates clearly the importance of short-range base-pairing by the comparison between completely inactive pHLL2644 and highly active
Discussion

All of the experimental data compiled here, from a complete set of single and complementary double nucleotide substitutions within the proximal influenza virus vRNA promoter element, are in agreement with a binary hooked conformation, as proposed in the ‘corkscrew’ model (Flick et al., 1996). This conformation is most likely realized during an initial step of promoter–polymerase interaction, i.e. recognition. While it has been demonstrated that polymerase attaches to the two vRNA ends in a step-wise manner (Li et al., 1998), the requirement for base-pairing within and in between both promoter sequence branches has been demonstrated through complementary base-pair exchanges, as shown for all three double-stranded elements. Promoter activity depends on the presence of two short-range base-pairs (2–9, 3–8) within either of the two proximal parts of this structure, while any long-range base-pairing in this region appears to be irrelevant. Long-range base-pairing between the vRNA 5′ and 3′ ends is required in the adjacent distal promoter element, however, and completes a group of three short double-stranded RNA stems that jointly constitute the structural core of the model. Since all of the base-paired nucleotides may be converted in complementary double substitutions into other base-pairs, a specific sequence requirement has only been identified at a few invariant positions, probably because of direct recognition by the virus polymerase. All of these nucleotides appear to be exposed in single-stranded 5′ and 3′ tetranucleotide loops supported by the two base-pairs in either of the two proximal stems: 5G, 7U, 7A(C). Therefore, the 5′ stem–loop structure, in particular, with its stable polymerase binding through entire rounds of transcription or replication (Lamb & Krug, 1996), would allow spatial recognition by polymerase at invariant loop residues (G5, A7). Double-stranded promoter elements may be recognized directly by their size and overall angular arrangement and indirectly by their contribution to stability and precise positioning of the loop nucleotides in the overall structural arrangement.

The 5′ and 3′ tetranucleotide loop sequences do not adhere to any of the standard tetranucleotide loop sequences (Wyatt & Tinoco, 1993) and an exchange, e.g. into 5′ GNRA (5′ AGAGGAAUC 3′), caused a severe reduction in promoter activity (14% relative activity; not shown). No activity was observed for a 3′ tetranucleotide UNCG construct. Therefore, the conformation of the vRNA tetranucleotide loop sequence appears to be different from that of a standard tetranucleotide loop and its constituent type of non-Watson–Crick base-pairing. However, specific external recognition of the two residues in the second and in the fourth positions of the influenza virus 5′ loop sequence (5G, 7A) appears to agree with the general properties of a standard tetranucleotide loop conformation (Valegard et al., 1994; Cate et al., 1996).

In the light of the substantial exchangeability of the promoter nucleotide sequence, as determined here exper-
mentally, it may be surprising that only a single substitution variant, U4C, has so far been observed in a small number of vRNA isolates, e.g. in influenza virus A/Puerto Rico/8/34 (Fields & Winter, 1982). The significance of this sequence alteration in wild-type influenza virus is still a matter of conjecture, but no influence was observed in the promoter-up system (Flick et al., 1996).

Recognition of an influenza virus vRNA molecule by viral polymerase and discrimination against all other RNA molecules in the infected cell appears to depend on (i) the three specifically recognized residues and (ii) the overall ‘corkscrew’ conformation, i.e. a conjunction of three divergently orientated RNA double strands in an oblique organization due to the unpaired angular residue A10 in the 5’ branch. Since residue A10 is not recognized specifically by itself but may be replaced by other nucleotides, it must be the specific conformational twist exerted upon the overall structure by its presence that aids specific recognition by viral polymerase, and is also involved in the packaging of VNP molecules.

A potential third element of recognition might be the very 5’- and 3’-terminal location of the promoter sequence, i.e. recognition of either or both of the vRNA 5’-phosphate and 3’-OH ends, in immediate conjunction with the ‘corkscrew’ structure. Several experiments have confirmed, however, that the vRNA promoter structure may be located further inside the RNA molecule and still be recognized (R. Flick & G. Hobom, unpublished results). This may render specific recognition of the vRNA termini a marginal element, at least for promoter-up variants. The promoter-adjacent non-coding sequences (beyond positions 16 and 15) are different for the eight segments and vary to some extent among individual isolates. In the promoter-up system used here, the regular non-coding sequences may be substituted for by any foreign recombinant sequences without a major influence on promoter activity or packaging. For wild-type promoter constructs, such variations and similar but not identical in structure. The conformational change during initiation of mRNA or cRNA promoter activity compared with vRNA promoter activity, a conclusion also supported by pHL1948 (stacking interactions between neighbouring G$^8$ and G$^9$) versus pHL2428 (no stacking interaction). Apparently, after polymerase binding in the ‘corkscrew’ mode, it is the facilitation of a conformational change into a ‘flat’ template structure that is required during initiation of transcription or replication in the presence of primer nucleotides. This change would be made easier by a less stable 3’ branch in the initial vRNA secondary structure. Only the 3’ branch has to undergo this conformational change during initiation of mRNA or cRNA synthesis, while the 5’ branch may stay unchanged and attached to polymerase throughout an entire round of mRNA synthesis (or even several rounds; Lamb & Krug, 1996). Promoter-up variant pHL1920, with the highest promoter activity, carries two A:U base-pairs at positions 3–8 and 3–5, without long-range complementarity. In contrast, base-pairing at positions 2 and 9 appears to be fundamental to forming the ‘corkscrew’ structure and the most stable interaction (G:C) is preferred in either branch of the overall structure.

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