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The Crystal Structure of a Cardiovirus RNA-Dependent RNA Polymerase Reveals an Unusual Conformation of the Polymerase Active Site

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ABSTRACT

Encephalomyocarditis virus (EMCV) is a member of the Cardiovirus genus within the large Picornaviridae family, which includes a number of important human and animal pathogens. The RNA-dependent RNA polymerase (RdRp) 3D pol is a key enzyme for viral genome replication. In this study, we report the X-ray structures of two different crystal forms of the EMCV RdRp determined at 2.8- and 2.15-Å resolution. The in vitro elongation and Vpg uridylylation activities of the purified enzyme have also been demonstrated. Although the overall structure of EMCV 3D pol is shown to be similar to that of the known RdRps of other members of the Picornaviridae family, structural comparisons show a large reorganization of the active-site cavity in one of the crystal forms. The rearrangement affects mainly motif A, where the conserved residue Asp240, involved in ribonucleoside triphosphate (rNTP) selection, and its neighbor residue, Phe239, move about 10 Å from their expected positions within the ribose binding pocket toward the entrance of the rNTP tunnel. This altered conformation of motif A is stabilized by a cation–π interaction established between the aromatic ring of Phe239 and the side chain of Lys56 within the finger domain. Other contacts, involving Phe239 and different residues of motif F, are also observed. The movement of motif A is connected with important conformational changes in the finger region flanked by residues 54 to 63, harboring Lys56, and in the polymerase N terminus. The structures determined in this work provide essential information for studies on the cardiovirus RNA replication process and may have important implications for the development of new antivirals targeting the altered conformation of motif A.

IMPORTANCE

The Picornaviridae family is one of the largest virus families known, including many important human and animal pathogens. The RNA-dependent RNA polymerase (RdRp) 3D pol is a key enzyme for picornavirus genome replication and a validated target for the development of antiviral therapies. Solving the X-ray structure of the first cardiovirus RdRp, EMCV 3D pol, we captured an altered conformation of a conserved motif in the polymerase active site (motif A) containing the aspartic acid residue involved in rNTP selection and binding. This altered conformation of motif A, which interferes with the correct positioning of the rNTP substrate in the active site, is stabilized by a number of residues strictly conserved among picornaviruses. The rearrangements observed suggest that this motif A segment is a dynamic element that can be modulated by external effectors, either activating or inhibiting enzyme activity, and this type of modulation appears to be general to all picornaviruses.

The Picornaviridae family is one of the largest virus families known and includes many important human and animal pathogens, such as polioviruses (PV), human rhinoviruses (HRV), and foot-and-mouth disease virus (FMDV) (1, 2). The Cardiovirinae genus consists of two species: encephalomyocarditis virus (EMCV) and related viruses (mengovirus, Columbia SK virus, and Maus-Elberfeld virus) (3) and Thievovirus (4–6). All of these viruses infect rodents, replicate in the gastrointestinal tract, and are transmitted by the fecal-oral route. Although rodents are the natural reservoir of these viruses, EMCV has also been isolated from many other hosts, including various mammals, birds, and invertebrates (7, 8). Infection of other mammalian species by EMCV is often fatal and associated with outbreaks of myocarditis and encephalitis. Human EMCV infection and associated disease have been documented, but clinical manifestation is probably infrequent (1). Thievovirus species are classified into five types, two of which infect humans: Vilyuisk human encephalomyelitis virus and Saffold virus (9, 10). Recently, several new types of Saffold
virus were isolated from young children with upper respiratory symptoms or gastroenteritis (11, 12; reviewed in reference 8).

The picornaviral RNA-dependent RNA polymerase (RdRp) 3Dpol, responsible for RNA genome replication, has been extensively studied, and there is a growing body of information available on 3Dpol structure and function. To date, the crystal structures of RdRps for six different members of the entovirus genus and for the aphthovirus FMDV have been reported either isolated or bound to different substrates (13–23). These enzymes adopt the classical closed right-hand architecture, having finger, palm, and thumb subdomains, with fingertips anchored on the thumb generating an encircled active site. Seven motifs (A to G) conserved in sequence and structure have been defined that play critical roles in recognition and binding of nucleoside triphosphates (A, B, D, and F), metal ion binding and phosphoryl transfer (A and C), structural integrity of the palm (D), priming nucleotide binding (E), and template binding (B, F, and G) (reviewed in references 24 and 25).

Mutational and structural analyses in PV also have demonstrated that some substitutions in residues located far from the active site, in particular at the polymerase N terminus, have significant effects on catalysis (14). In addition, a number of mutant polymerases that show altered polymerization fidelity have been identified harboring mutations remote from the active site (26–28). All of these observations suggest that nucleotide binding and incorporation are modulated by a long-distance network of interactions (14, 29–32).

To gain insight into the elucidation of the dynamics-function relationships in RdRps, we have solved the crystal structure of the first cardiovirus RdRp, the EMCV 3Dpol, in two different crystal forms at 2.15-Å and 2.8-Å resolution. Interestingly, in one of the crystal forms, we captured an unusual conformation of motif A, occupying the NTP channel entry. In this conformation, motif A directly interacts with a conserved lysine in the finger domain and with motif F. The structure also shows that the first residue, Gly1, is moved out of its binding pocket, buried in the finger domain, toward an exposed orientation in the polymerase surface. Finally, we have also demonstrated that the recombinant enzyme is active in vitro for both Vpg uridylylation and RNA elongation.

MATERIALS AND METHODS

Protein expression and purification. The genomic region coding for the RNA-dependent RNA polymerase 3D of encephalomyocarditis virus, type mengovirus (EMCV 3Dpol), derived from infectious clone pM16.1 (33), was cloned into the expression plasmid pETG20A Gateway (Invitrogen). This plasmid provides a six-histidine tag attached at the carboxyl terminus of the protein which can be removed by digestion with tobacco etch virus (TEV) protease (Invitrogen).

Protein was produced in Escherichia coli Rosetta (DE3) pLYS strain. Transformed cells were grown in Turbo Broth medium (AthenaES) supplemented with 100 μg ml−1 ampicillin and 34 μg ml−1 chloramphenicol at 310 K until an optical density at 600 nm (OD600) of 0.4 to 0.6 was reached, and then the solution was tempered to 293 K. Expression was induced with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 293 K. Cells were pelleted by centrifugation at 4,500 × g for 30 min and then resuspended in 40 ml of phosphate-buffered saline (PBS; 1×). Finally, cells were harvested by centrifugation and stored at −80°C.

Cells were resuspended in a cold lysis buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.1% (vol/vol) Triton X-100, 10% glycerol, 1 mg ml−1 lysozyme, and 10 μg ml−1 DNase and lysed by sonication. Cellular debris were pelleted by centrifugation, and the soluble fraction was filtered and loaded into a 5-ml HisTrap HP (GE Healthcare). Elution was carried out with an imidazole gradient (10,000 μM weight cutoff polyethersulfone [PES]; Millipore) previously equilibrated with a protein buffer containing 50 mM arginine and 50 mM glutamine (Sigma-Aldrich). The protein was stored at 193 K and the purity grade analyzed by SDS-PAGE.

For the biochemical activity assays, the EMCV 3Dpol was expressed at 17°C in Turbort broth (Arcadia Biotech). Protein purification and tag removal were performed under non-denaturing conditions as previously described (34). The final size-exclusion chromatography step was performed in 10 mM HEPES, 300 mM NaCl, 2 mM DTG, pH 7.5.

Polymerase activity assays. The polymerase elongation activity assays were performed as described previously (16). Starting conditions for filter-binding polymerase activity assays optimized for coxsackievirus B3 (CVB3) 3Dpol were conducted in a mix of 50 μl containing 50 mM Tris, pH 7.0, 10 mM KCl, 0.8 mM MgCl2, 9% glycerol, 2 μM oligo(dT15) (Invitrogen) annealed to 350 nM poly(rA) [termed poly(rA)/dT15] (Invitrogen) at 37°C for 10 min, 500 μM poly(rA), 0.5 μCi [3H]UTP (35.5 Ci/mmol; GE Healthcare) and 40 nM EMCV 3Dpol, 10 μM UTP, and 0.5 μCi [3H]UTP (35.5 Ci/mmol; GE Healthcare). Final conditions obtained for EMCV 3Dpol were 50 mM morpholine propane sulfonic acid (MOPS), pH 7.0, 10 mM KCl, 4 mM MgCl2, 9% glycerol, 2 μM oligo(dT15) annealed to 350 nM poly(rA), 0.5 μM EMCV 3Dpol, 100 μM UTP, and 2 μCi [3H]UTP (35 Ci/mmol). Visualization of elongation products by denaturing PAGE was carried out as described previously (16). The same reaction mixture was used as described above, except that [35S]UTP (10 μCi) was used instead of [3H]UTP. Ten-μl samples were taken at 0, 10, 20, and 30 min.

VPG uridylylation reactions were performed as described previously (16). The reaction mix for EMCV 3Dpol contained 50 mM HEPES, pH 8.0, 10 mM KCl, 1 mM MnCl2, 9% glycerol, 2 μM poly(rA), 150 μM EMCV VPg (GPYNETTRIKPTKTLQDQ; Perbio Science, France), 2 μM EMCV 3Dpol, 5% dimethylox sulfone (DMSO), 100 μM UTP, and 20 μCi [35S]UTP. Fifteen-μl samples were taken at 0, 60, and 120 min and analyzed by Tris-tricine SDS-PAGE.

Crystallization. Initial crystallization trials of EMCV 3Dpol were performed by the sitting-drop vapor diffusion method at both 277 and 293 K in 96-well Greiner plates using a nanoliter-drop crystallization robot (Cartesian Microsys 4000 XL) and the commercial screens: crystal screens 1 and 2 and salt RX (Hampton research). Protein solution (10 mg ml−1 in 500 mM NaCl, 50 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM DTT, and 1 mM EDTA) droplets of 100 nl were mixed with 100 nl of precipitant solution; the reservoir volume was 100 μl. Two different crystal forms were obtained. The first crystals appeared in 1 day at 277 K from a solution containing 10% polyethylene glycol (PEG) 6,000, and 2.0 M NaCl. These crystals were optimized in the same plates using the Cartesian robot but increasing the drop volume to 400 nl (200 nl protein solution and 200 nl crystallization buffer). The second crystal form was optimized at 293 K in a 24-well Greiner plate in crystallization drops containing 1 μl of protein solution and 1 μl of crystallization buffer (4.0 M ammonium acetate and 0.1 M sodium acetate, pH 4.6) equilibrated against 1 ml of crystallization buffer. Crystals appeared in 4 or 5 days.

X-ray diffraction analysis. EMCV 3Dpol data sets were collected at the ALBA-CELSYS synchrotron light facility (Barcelona) on a Pilatus 6M Decris detector at beamline XALOC (λ = 0.979 Å). All of the data sets were recorded at 100 K. The first crystal form, belonging to the 14,22 space group, diffracted to 2.15-Å resolution (Table 1). Two hundred images
were collected with an oscillation angle of 0.5° and 1-min exposure time as a collection strategy. The second crystal form, belonging to the space group C2 and with six independent RdRp molecules present in the asymmetric unit, was collected with an oscillation angle of 0.5° and 1-min exposure time as a collection strategy. The second crystal form, belonging to the space group C2 and with six independent RdRp molecules present in the asymmetric unit, was collected with an oscillation angle of 0.5° and 1-min exposure time. Diffraction images were processed with XDS (35) and internally scaled with SCALA (CCP4i) (36).

**Table 1: Data collection and refinement statistics**

<table>
<thead>
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<th>Parameter</th>
<th>Value(s) for crystal form:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolution range (Å)</td>
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</tr>
<tr>
<td>Space group</td>
<td>I4,22</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>122.55, 122.55, 198.79</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>90, 90, 90</td>
</tr>
<tr>
<td>Rmerge</td>
<td>0.09 (0.77)</td>
</tr>
<tr>
<td>I/</td>
<td></td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.4 (98.3)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.0 (5.0)</td>
</tr>
<tr>
<td>Refinement results</td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>43.15–2.15</td>
</tr>
</tbody>
</table>

**Structure solution and refinement.** In both space groups, the structure of the EMCV 3Dpol was determined by molecular replacement with PHENIX software (37) using a search model derived from the structure of FMDV polymerase (Protein Data Bank [PDB] entry 1U09). The correctly oriented and positioned models were manually rebuilt using Coot (38) and automatically refined with Refmac5 (39) for the I4,22 structure; Refmac5 and PHENIX were used for the C2 structures. Noncrystallographic symmetry (ncs) restraints were initially applied to the six independent molecules present in the asymmetric unit of the C2 crystals using Refmac5. However, the ncs restrained-refinement result was unstable because the presence of different conformations in many polymerase regions, especially in the surface-exposed loops, was affected by crystal-packing interactions. Therefore, the variable regions were manually rebuilt using Coot, and the final cycles of automatic refinement were performed with PHENIX. The refined structures in both space groups contain the complete protein residues (from Gly1 to Thr460). The C2 structure also contains 7 glycerol molecules and 123 water molecules, and the I4,22 structure contains 2 glycerol molecules and 105 water molecules (Table 1). In addition, an elongated extra density, present in the active-site cavity of the I4,22 crystal form, has been interpreted according to the presence of a glutamine molecule from the buffer used for washing the concentrator membranes prior to protein concentration (40). The statistics of the refinement for the two structures are summarized in Table 1.

**Structural analysis.** The knowledge-based potentials of the program Prosa2003 (41) were used to compare the quality of the structures. Prosa potentials are based on the use of the inverse of the Boltzmann equation, inferring the energy of a system based on the frequency with which pairs of residues are found at a given distance in the Protein Data Bank. The total energy is split into two main components: pair energy, which checks the quality of the residue-residue interactions, and surf energy, to check the residue interaction with the solvent. The quality of the whole structure is evaluated by means of the Z score (Z score = (E – <E>)/σ, where E is the energy of a particular conformation, <E> is the average of energies obtained with the sequence tested in all possible folds stored in Prosa, and σ is the standard deviation of the test). The combined (comb) Z score for surf energy and pair energy potentials for the EMCV 3Dpol structures yielded identical values for both standard and altered conformations of motif A (Z score [comb] = –13.5), with subtle differences in the individual surf and pair energies calculated (Z score [surf] = –10.03 and Z score [pair] = –10.45 for the standard conformation; Z score [surf] = –9.92 and Z score [pair] = –10.64 for the altered conformation). These values were within the expected range for a protein of 460 residues (Prosa Manual; [http://www.came.sbg.ac.at], indicating that both standard and altered conformations were stable.

However, these energy calculations are not enough to ensure that the conformation of a sequence in a particular fold is the best. To further check the local differences in stability between the two motif A loop conformations, a new analysis was performed taking into account only the residues of the motif A loop (237 to 243), the closest contacting zone that included the region of residues 50 to 64 and residue 1. Calculations were done by scanning the sequence of these regions with all possible amino acid substitutions. The Z-score of the new structure was calculated and compared to the Z-score of the original (nonmutated) sequence. The results obtained are summarized in Table 2.

**PDB accession numbers.** The PDB accession codes for the EMCV 3Dpol structures are 4NYZ and 4NZ0 for I4,22 and C2 crystal forms, respectively.

**RESULTS**

**In vitro polymerase activity of recombinant EMCV 3Dpol.** Polymerase activity of the purified EMCV 3Dpol was assayed by measuring elongation of an oligo(dT15) primer hybridized to a poly(rA) template through incorporation of [3H]UTP. Negligible elongation activity could be observed when using conditions optimized for CVB3 3Dpol ([50 mM Tris buffer, pH 7.0, 0.8 mM MgCl2, 40 mM 3Dpol, 9% glycerol, 10 μM UTP, 0.01 μM [3H]UTP, 2 μM oligo(dT15)] annealed to 350 nM poly(rA); L. van der Linden, L. Vives-Adrián, B. Selisko, C. Ferrer-Orta, H. Venceslao, K. Lanke, A. M. De Palma, F. Tanchis, N. Goris, D. Lefebvre, K. De Clercq, P. Meyssen, C. Lacroix, G. Pürrstinger, R. Ulferts, B. Coutard, B. Canard, N. Verdaguier, J. Nepts, and E. F. J. M. van Kuppevelt, unpublished data). Therefore, the assay was optimized for EMCV 3Dpol using conditions described for FMDV 3Dpol (42), an enzyme with a higher similarity to EMCV 3Dpol than CVB3 3Dpol, as a guideline. A higher enzyme concentration (0.4 to 1 μM compared to 40 nM for CVB3 3Dpol) was required to observe some activity (data not shown). The change to MOPS buffer (pH 7.0) instead of Tris (pH 7.0) was decisive (Fig. 1A). This was also the
Overall architecture of the EMCV RdRp. Two different crystal forms, space groups I4122 and C2, were obtained from the EMCV 3Dpol (Table 1; also see Materials and Methods). The C2 crystals diffracted to 2.8-Å resolution and contained six independent polymerase molecules in the asymmetric unit. The structures were solved by molecular replacement using a homology model based on the structure of FMDV 3Dpol (PDB entry 1U09) as a search model. Data collection and refinement statistics are summarized in Table 1.

EMCV 3Dpol adopts the classical right-hand conformation of RdRps with the N terminus, finger, and thumb subdomains interacting to encircle the active site (Fig. 2A). In the C2 crystals, the six independent polymerase molecules show similar conformations (the root mean square deviation [RMSD] between all Ca atoms is 0.5 Å). The main differences are located at the polymerase N terminus, residues 41 to 64, and in the palm loops, residues 292 to 297 and 360 to 368, at the N and C termini of motifs B and D, respectively (Fig. 2C). Subtle differences are also seen in the region of residues 440 to 448, joining the thumb helices (Fig. 2B).

A summary of the residues of the motif A loop (residues 237 to 243) and the surrounding selected residues (50 to 64 and 1) is shown in Table 2. We used Z-scores of pair, surface, and combined energies. Columns show the number of mutations stabilizing (Δ < 0) and destabilizing (Δ > 0) the standard conformation of EMCV 3Dpol pseudoenergies. Δ is the difference between the mutated residue and initial residue energies. By convention, we assumed that when Δ < 0 is greater than 15, a significant unstable localization of the conformation occurred. Column I shows a plus mark when the mutations stabilize the conformation in a particular position. Rows show the selected residues for this analysis.

**TABLE 2** Pseudoenergies of selected residues of I4122 and C2 EMCV 3Dpol conformations

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<th>Residue</th>
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<td></td>
<td>I4122</td>
<td>C2</td>
<td>I4122</td>
</tr>
<tr>
<td></td>
<td>Δ &lt; 0</td>
<td>Δ &gt; 0</td>
<td></td>
</tr>
<tr>
<td>No.</td>
<td>Identity</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>G</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td>50</td>
<td>D</td>
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<tr>
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active site, as reported for all other picornaviral 3D<sub>pol</sub> structures
determined to date (Fig. 3A and B). However, in the sixth mole-
cule, the Gly1 binding residues Thr58 and Asn60 are reoriented
and participate in packing contacts with a neighboring molecule in
the crystal, leaving Gly1 in a partially exposed position (Fig. 3C).
This glycine is clearly visible in the electron density maps, and its posi-
tion is probably stabilized by a salt bridge created between the
Gly1 N terminus and the side chain of Glu4 (Fig. 3C).

As for the other picornaviral RdRps, the EMCV 3D<sub>pol</sub> fingers
are divided into two regions: an inner region that consists primar-
ily of a bundle of α-helices (from α1 to α4 and α6, α8, and α9),
packed against the palm subdomain, and an outer region, consist-
ing of a five-stranded mixed β-sheet (β1, β5, β7, β9, and β10),
with strand β1 contributed from the N terminus. The long region
connecting strands β5 and β7 (residues 151 to 179) contains two
small secondary structural elements, α5 and β6, which, together

FIG 1  In vitro polymerase activity of EMCV 3Dpol. (A to D) In vitro EMCV 3Dpol elongation activity measuring incorporation of [3H]UTP using poly(rA)/dT<sub>15</sub>
as the template-primer. (A to C) Elongation activity of EMCV 3Dpol while varying cofactor MgCl<sub>2</sub>/MnCl<sub>2</sub>, UTP, and glycerol concentrations and the type of buffer. The initial velocity of the reaction (V<sub>i</sub>) represents the polymerase elongation activity. The V<sub>i</sub> observed with the starting conditions for the optimization was set to 100%. (D) Elongation activity of EMCV 3Dpol using the optimized conditions. (E) EMCV 3Dpol elongation activity as visualized by denaturing PAGE. The dT<sub>15</sub> primer is elongated with ([32P])UTP using a poly(rA) template. (F) VPg uridylylation assay. In the presence of a poly(rA) template, [32P]UTP is
covaently coupled to the primer VPg by EMCV 3Dpol. Reaction products were analyzed using Tris-tricine-SDS-PAGE.
with the $3_{10}$ helix $\eta_1$ and the strand $\beta_2$ at the N terminus, extend toward the thumb domain, forming the fingertip region (Fig. 2A). The long $\beta_5$-$\beta_7$ loop also contains the conserved motif F, which is rich in positively charged residues that form the roof of the rNTP entry channel (Fig. 2A). These residues bind the incoming rNTP and also assist the reorientation of its triphosphate moiety in the correct position for catalysis, as seen in the structures of enterovirus and aphthovirus catalytic complexes (17, 19). Finally, at the front side of the fingers, the long loop connecting helices $\alpha_3$ and $\eta_2$ (residues 98 to 120) contains motif G (residues 110 to 120), which forms the template channel entrance (Fig. 2A).

The palm domain of EMCV 3Dpol consists of the central three-
which form the interface with the mostly helical thumb (Fig. 2A). The small size of this domain contributes to the formation of the large central cleft, located in the front of the molecule, that is typical of primer-dependent RdRps (21, 43, 44). The end of the C-terminal helix \( \alpha_{15} \) is anchored to the palm domain via hydrophobic interactions between Phe459 and Val216 of helix \( \alpha_{7} \) and Leu390 in the turn of motif E. The most C-terminal residue, Trp460, appears exposed to the solvent and participates in interactions with neighboring 3Dpol molecules in the crystal. Mutational studies in mengovirus indicated that a deletion of Trp460 or its replacement by Ala or Phe dramatically impaired viral RNA replication. The study also showed that replacement of this Trp by Tyr or His did not alter viral growth. In light of these data, the authors suggested that Trp460 is involved in thumb-palm contacts via a hydrophobic interaction with Val216 and a polar bond with the side chain of Thr219 (45).

The helix \( \alpha_{10} \) also contains the conserved Asn302, which, together with Asp240 in motif A, has been described to be essential for selection of rNTPs over deoxynucleoside triphosphates (dNTPs) by hydrogen bonding to the 2’ hydroxyl group of the incoming nucleotide. In the six independent RdRp structures seen in the C2 crystals, Asn302 appears to be hydrogen bonded to Asp240 (Fig. 3B and C), as in other picornaviral RdRp structures. The helix \( \alpha_{11} \) supports the central (\( \beta_8, \beta_{11}, \) and \( \beta_{12} \)) sheet and, together with the long loop at its C terminus (\( \alpha_{11}-\beta_{13} \) loop), forms motif D. The transition between palm and thumb subdomains is provided by the two antiparallel \( \beta \)-strands (\( \beta_{13} \) and \( \beta_{14} \)), forming the structural motif E (Fig. 2A).

In common to all other picornaviral RdRps, EMCV 3Dpol possesses a small thumb subdomain, built mainly by helices \( \alpha_{12} \) to \( \alpha_{15} \), organized in a four helix-bundle (Fig. 2A). The overall 3Dpol structures observed in the two different space groups also agree very well (RMSD of 1.1 Å) (Fig. 2B). However, an important distortion of the polymerase active site in different 3Dpol structures. (A) PV 3Dpol bound to an rNTP molecule shown in yellow (PDB entry 1RA6); (B) EMCV C2 crystals, representative structure of five of the six independent molecules in the asymmetric unit; (C) EMCV C2 crystals, sixth molecule; (D) EMCV I4\( \bar{1} \)22 crystals. In all panels, the polymerase residues are shown as sticks in atom type code, with the motif A Asp highlighted in red and the motif B Asn shown in blue. The rNTP found in the PV structure is modeled in EMCV structures by superposition of the active-site residues and represented in semitransparent yellow sticks.
The rearrangement mainly affects the polymerase motif A residues from Tyr236 to Ala246, where Asp240, the amino acid in charge of rNTP selection, and the neighboring Phe239 move 10 Å away from its position in the catalytic cavity of active RdRp, directed toward the entrance of the nucleotide channel and approaching motif F (Fig. 4F and C). This altered conformation of motif A is mainly stabilized by a cation–π interaction established between the phenyl ring of Phe239 and the ε-amino group of Lys56 located in the finger domain at the C-terminal end of helix α1 (Fig. 4D). The Phe239 side chain is also involved in strong hydrophobic contacts with residues Lys172 and Arg163 in motif F (Fig. 4D and E). Additional interactions contributing to the stabilization of motif A in the altered position are observed between the side chain of residue Thr242 and the finger residue Asn60 and between main and side chains of His243, Leu291, and Asn306.
within motif B (helix α10) (Fig. 3D). In this altered position of motif A, the active-site residue Asp240 remains totally exposed in the rNTP tunnel edge (Fig. 4B).

The movement of motif A is associated with other conformational changes in the neighboring finger region: from Phe54 to Ser63 (C terminus of the α1 and α1-α2 loop) (Fig. 2B), which directly interacts with motif A in both standard and altered conformations (Fig. 3B, C, and D), and at the 3Dpol N terminus, where the very first Gly1 residue moves from its binding site anchored in the finger domain toward a totally exposed orientation in the polymerase surface (Fig. 3D).

In addition, the drastic movement of the motif A loop leaves an empty space in the palm site, particularly where Phe239 used to be packed (Fig. 5C and D). In the structure, this space is filled by the presence of a density which has been interpreted as a glutamine molecule coming from the washing buffer of the concentration filters (see Materials and Methods). A number of water molecules are also found in this region. Finally, the Asn302 and Asn306 side chains have been changed in their rotamer conformation and interact with one another and with Tyr236, partially occupying the Phe239 ring (Fig. 5D).

Structural comparisons have also detected other polymerase
structures of various picornaviral replication-elongation complexes, trapped in different steps of the catalytic process, have revealed a mechanism of active-site closure that does not require large interdomain rearrangements (17–20). Structural comparisons evidenced that 3Dpol uses subtle conformational changes in the palm domain, facilitating the interactions with the ribose hydroxyl groups of the incoming rNTP. In particular, the side chain reorientations in the conserved aspartic acid of the motif A C terminus (Asp240 in EMCV) and in motif B residues Asn302 and Ser293 are responsible for rNTP recognition and binding (19, 43, 44). Furthermore, a number of basic residues of motif F were also involved in binding the triphosphate moiety of the incoming nucleotide (Fig. 2D and E). After the correct accommodation of the rNTP substrate in the active site, a fine shift of motif A positions the catalytic aspartic acid, located at the N terminus of the motif, in the correct orientation for metal binding (Fig. 2E). A number of catalytic complexes have been crystallized in an initial precatalytic closed conformation, where the β-strand structure of motif A was completely formed, allowing catalysis to take place by the common two-metal-ion mechanism (17, 18) (Fig. 2E). These local changes serve to fully structure the active site upon correct nucleotide binding. As picornaviral RdRps do not possess any proofreading activity, the mentioned rearrangements associated with the catalytic cycle seem to be the major fidelity determinants of these enzymes (reviewed in references 50 and 44). Besides these fine movements, recent molecular dynamics and nuclear magnetic resonance data have provided evidence that the motif D loop is highly dynamic and that the movements of this region serve to close the active site when the correct nucleotide is bound (31, 51, 52). This motif contains a strictly conserved lysine, which appears to be the proton donor for the pyrophosphate leaving group after nucleotide incorporation (53). Pyrophosphate protonation is not essential but contributes 50-fold to 1,000-fold to the rate of nucleotide addition (54). Therefore, motif D and, in particular, the dynamics of this conserved lysine appear to comprise an important element affecting both fidelity and polymerization efficiency (31, 51, 53–55).

The six EMCV 3Dpol-independent molecules found in the C2 crystals showed an open conformation of the active site, as expected for the apo-form of the enzyme. In contrast, the 14,22 crystals trapped the EMCV enzyme in a closed-like conformation, with the β-sheet supporting motif A totally formed and the catalytic Asp235 positioned in front of the motif C Asp333 (Fig. 4B and C and 5B). This active-site conformation has never been observed before in the absence of RNA and a correctly base-paired NTP. However, the 14,22 structure shows an unprecedented movement of the motif A loop harboring the rNTP binding residue Asp240 −10 Å away from the active site. In this orientation, Asp240 occupies the edge of the NTP entry tunnel (Fig. 4B and C). In addition, all of the residues expected to participate in the EMCV active-site closure during catalysis were involved in contacts stabilizing the altered conformation of the A-loop (Fig. 4C to E). Next to Asp240 is the phenyl ring of Phe239, which forms a π-cation interaction with Lys56 and is further stabilized by hydrophobic interactions with the side chains of Lys172 and Arg173 of motif F (Fig. 4D). Moreover, the main chains of residues Gly290,
Leu291, Ile230 (motif B), and Asp334 (motif C) also participated in contacts with the altered motif A (Fig. 4D and E). Finally, in this structure the Asp240 interacting residue, Asn302, together with Asn307 appeared hydrogen bonded to the catalytic Asp333 of motif C, which had changed its rotamer conformation (Fig. 5A to D).

The large movement of motif A seems to be associated with other important changes in the molecule. In particular, rearrangements were located at the finger residues 54 to 63 and at the polymerase N terminus. All picornavirus 3D<sub>p</sub> structures reported to date have shown the N-terminal glycine buried in a pocket at the base of the finger region, participating in a network of hydrogen bonds with other highly conserved glycine residues in finger and palm domains (Fig. 3A and B) (14, 29, 56). This network of interactions helps the correct positioning of the rNTP binding Asp residue in the active site (14). In enterovirus and aphthovirus, the amino group of Gln1 establishes hydrogen bonds with the backbone carbonyls of three conserved glycines, one at the fingers and the last two within the motif B loop, in an almost perfect tetrahedral geometry (Fig. 3A). The conformation was further stabilized by other main-chain hydrogen bonds formed between residues of motifs B and A (Fig. 3B). Previous mutational, biochemical, and structural data on PV showed that the 3D<sub>p</sub> activity was extremely sensitive to mutations at the N terminus (14, 57, 58). This sensitivity, as well as the lack of polymerase activity in the PV precursor 3CD protein, was thought to be associated with the removal of the 3D<sub>p</sub> N terminus from its binding pocket and, as a consequence, to an alteration of the pattern of interactions mentioned (29).

In the standard conformation of motif A, Phe239, the amino acid that has experienced the most dramatic rearrangement (Fig. 4 and 5), appears tightly packed to the aromatic residues Tyr236 and His243 within the same motif and makes additional hydrophobic contacts to Ile309 (helix α10 of motif B) and, to a lesser extent, to Tyr357 (motif D loop). In addition, its main-chain oxygen is hydrogen bonded to the His243 side chain, which is also involved in other two polar interactions with the side chains of Asn302 and Tyr357 (Fig. 5C). In the altered structure of motif A, the His243 side chain has changed its rotamer conformation and appears to participate only in a hydrogen bond with the reorganized Asn306 side chain (Fig. 5D). Comparisons to other picornaviral polymerases of known structures demonstrate the high conservation of this palm region (Fig. 5C, E, and F). The equivalent position of Phe239 is always occupied by an aromatic residue in picornaviral polymerases, with Phe in cardiovirus and aphthovirus and Tyr in most enteroviruses. In all of the enterovirus 3D<sub>p</sub> structures solved, the OH group of this Tyr side chain appears to be hydrogen bonded to a main-chain residue of motif B, being an additional element that stabilizes the motif A loop in the standard conformation (Fig. 5D). The Phe239-interacting residue Tyr236 is also strictly conserved among picornaviruses; however, His234 is only conserved in the cardio- and aphthovirus genera. In FMDV 3D<sub>p</sub> structures solved, both in absence and in the presence of RNA (19, 20), the equivalent His residue is involved in similar contacts (Fig. 5). In addition, whereas both FMDV and EMCV C2 crystals have been obtained at acidic pH, the EMCV I4,22 crystals have grown at pH 8 (19, 20; also see Materials and Methods). Taking into account the different pH conditions used in crystal growth and the pattern of interactions described above, we tentatively speculate that the pH differences affect the protonation state of His243, affecting the interactions mediated by this residue and, as a consequence, preventing or facilitating the large conformational change of the motif A loop. The altered conformation of motif A leaves an empty space in the palm that might be occupied by small molecules, like the glutamine molecule found in the 14,22 structure, which probably contribute to the stabilization of this altered conformation.

In conclusion, the structural data presented here suggest that the motif A loop would act as a negative regulator of RNA synthesis, interfering with the access of the incoming nucleotides to the active site. The role of this negative regulation is not known. However, the participation of highly conserved residues in maintaining the altered form of motif A, especially in cardio- and aphthoviruses, suggests that this conformational change is general at least to the polymerases of these two picornavirus genera. The presence of flexible elements in the polymerase regions that play roles in template, primer, or nucleotide binding is essential for its function. The dynamics of these elements can be modulated by external effectors, either activating or inhibiting the enzyme activity. Small-molecule compounds designed to stabilize altered motif A would prevent the correct positioning of the incoming rNTP to the active site and, as a consequence, the polymerization function in picornaviruses.

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