Domain Structure of the Vaccinia Virus mRNA Capping Enzyme

EXPRESSION IN ESCHERICHIA COLI OF A SUBDOMAIN POSSESSING THE RNA 5'-TRIPHOSPHATASE AND GUANYLYLTRANSFERASE ACTIVITIES AND A KINETIC COMPARISON TO THE FULL-SIZE ENZYMEE

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The RNA 5’-triphosphatase, nucleoside triphosphate phosphohydrolase, and guanylyltransferase activities of the vaccinia virus mRNA capping enzyme were previously localized to an NH2-terminal 60-kDa domain of the D1R subunit. Measurement of the relative ATPase and guanylyltransferase activities remaining in D1R carboxy-terminal deletion variants expressed in Escherichia coli BL21(DE3)pLyS5 localizes the carboxyl terminus of the active domain to between amino acids 520 and 545. Failure to obtain a deletion mutant with the loss of one activity indicates that the catalysis of both reactions requires a common domain structure. Based on these results, a truncated D1R protein terminating at amino acid 545 was expressed in E. coli and purified to homogeneity. D1R 1-545 was found to be kinetically equivalent to the holoenzyme in regard to ATPase, RNA 5’-triphosphatase, and guanylyltransferase activities. Measurement of RNA binding by mobility shift and UV photo-cross-linking analyses also demonstrates the ability of this domain to bind RNA independent of the methyltransferase domain, comprised of the carboxyl terminus of D1R from amino acids 498–844 and the entire D12L subunit. RNA binding to D1R1-545 is substantially weaker than binding to either the methyltransferase domain or the holoenzyme. Binding is inhibited by 5’-OH RNA and to a lesser extent by DNA oligonucleotides in a concentration dependent manner which correlates with the inhibition of RNA 5’-triphosphatase activity by these same oligonucleotides. We conclude that D1R1-545 represents a functionally independent domain of the mRNA capping enzyme, fully competent in substrate binding and catalysis at both the triphosphatase and guanylyltransferase active sites.

Vaccinia virus, a member of the Poxviridae family, is a double-stranded DNA containing virus that carries out its replication entirely within the cytoplasm of the infected host cell (for a review, see Ref. 1). This life cycle necessitates that most enzymes required for gene expression and DNA replication are encoded by the virus. Many of these enzymes are packaged into the virion and are active in the expression of early viral genes, immediately upon infection. These enzymes include a multi-subunit RNA polymerase (2, 3), early gene transcription initiation (4, 5), and termination factors (6), an mRNA capping enzyme (7–9) and a poly(A) polymerase (10, 11).

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1 The abbreviations used are: ppp(N)ₙ, RNA; D1R, mRNA capping enzyme large subunit; D12L, mRNA capping enzyme small subunit; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PEI, polyethyleneimine; TBE, Tris borate EDTA; PCR, polymerase chain reaction; HT, hydroxyapatite.
appears to be sufficient for both guanylyltransferase and phosphohydrolase activities. This portion of D1R contains the lysine residue at position 260 (27, 38) required for the formation of the covalent enzyme–GMP intermediate of the guanylyltransferase reaction (29, 30, 41). The question arises whether the phosphohydrolase and guanylyltransferase activities in the 60-kDa NH₂-terminal D1R portion of the capping enzyme may be functionally separable into two independent domains. The domain structure of the vaccinia virus enzyme is analogous to the capping enzymes purified from rat liver (42) and brine shrimp (43) in which a single protein possesses both guanylyltransferase and RNA 5'-triphosphatase activities. In the latter enzyme, the guanylyltransferase and RNA 5'-triphosphatase activities have been fractionated by limited tryptic digestion into 44- and 20-kDa protelysis products, respectively, separable by gel filtration chromatography (43). Full capping activity, i.e. the ability to employ triphosphate-ended RNA as a substrate for guanylylation, could be reconstituted in vitro by combining the two functional subdomains. In the yeast mRNA capping enzyme, the separation of the RNA 5'-triphosphatase and guanylyltransferase active site is more distinct as the two activities are present in separate subunits, encoded by different genes (31, 32). These latter two examples argue that the existence of two catalytic subdomains in vaccinia D1R 60-kDa domain is possible.

In this report, we describe in greater detail the structure-function relationship of the D1R 60-kDa subdomain. Expression in E. coli of carboxyl-terminal deletions in the D1R gene demonstrates a shared functional domain for the RNA 5'-triphosphatase and guanylyltransferase catalytic sites. A kinetic characterization of the purified D1R-1–545 domain demonstrates a functional equivalence to the holoenzyme in regard to RNA 5'-triphosphatase, ATPase, and guanylyltransferase activity. This domain also possesses the ability to bind triphosphorylated RNA independent of the methyltransferase domain demonstrating that the binding of RNA to this domain alone is sufficient for catalysis at the RNA 5'-triphosphatase and guanylyltransferase active sites.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**

Construction of Plasmids Directing the Synthesis of Carboxyl-terminal Deletions

The structures of the expression plasmids pET3a D1R-63, pET8c D1Nco and pET3a D1R/D12L are described elsewhere (23). To generate a family of plasmids capable of expressing a set of carboxyl-terminal deletions of D1R, pET3a D1R-63 was linearized at a single Ncol site at position 2234 and the DNA digested with Bal-31 exonuclease for varying times at 37 °C, followed by S1 nuclease treatment. The resulting products were end-filled with the Klenow fragment of DNA polymerase I, ligated, and used to transform E. coli TB1, HMS174(DE3), and BL21(DE3)plyS (33) for the synthesis of a family of nested D1R carboxyl-terminal deletions. The carboxyl terminus of each protein product was determined by DNA sequence analysis of each pET3a D1R/D12L encoded subunit.

**DNA Synthesis and Purification—** The plasmid pET8c D1R-1–545 which directs the synthesis of D1R-1–545 was constructed (Fig. 1). A DNA fragment (term 168) corresponding to a portion of the D1R sequence from position 1501 to 1737 (including the EcoRI site at position 1546) was amplified by PCR. The 3' end of this fragment was modified by introducing two translation stop codons in frame beginning at position 1738 of D1R (34) using a leftward primer (5'–GGG AGA TCT TTA TTA ATT ATT GGC GTA TTG AT < 3') containing a BglII restriction site at its 5' end for ease of subcloning. The rightward primer was complimentary to D1R sequence beginning at position 1501. The term 168 PCR fragment was subcloned into the D1R expression plasmid pET8c D1Nco by ligation of three DNA fragments including: the EcoRI to PstI fragment of the parental plasmid pET8c D1Nco, the PstI and BamHI fragment of pET8c, and the EcoRI to BglII term 168 fragment. The ligation products were used to transform E. coli HMS174(DE3), BL21(DE3), and BL21(DE3)plyS.

**RNA Synthesis**

Templates for RNA Synthesis—The large-scale in vitro synthesis of specific oligonucleotides is described elsewhere (24). Briefly, RNA was synthesized by the transcription of linearized plasmid DNA templates by T7 RNA polymerase. To produce a transcript 23 nucleotides in length, the following plasmid was constructed. An oligonucleotide with the sequence AATTAATATTTTG was synthesized and annealed to an oligonucleotide of the complimentary sequence. The resultant double-stranded oligonucleotide, yielding AATT 5' overhangs, was cloned into the EcoRI site of pGEM32(+) . Transcription of this plasmid linearized at the EcoRI site yielded a 23-mer with the sequence PPPGGC-GAAUUAAUAUUUGAAUU. Transcription of pGEM32(+) –linearized at the HindIII restriction site yielded RNA 60 nucleotides in length, corresponding to the multiple cloning site sequence.

**RNA Synthesis and Purification—** For the synthesis of α-32P-labeled RNA employed as a substrate for RNA 5'-triphosphatase assays, transcription reactions contained 400 μl of linearized plasmid DNA, 1 mM ATP, CTP, and UTP, 0.5 mM GTP, 1 mCi of [α-32P]GTP (DuPont NEN, 3000 Ci/mmol) and 300–500 units of T7 RNA polymerase per ml of reaction. Transcription reactions were carried out for 2 h at 37 °C, at which time 100 units of RNase-free DNase I (Promega) was added (0.25 unit/μg of DNA) and incubated for an additional 30 min at 37 °C. The reaction was quenched, and protein was extracted by adding an equal volume of 24:24:1 phenol–STE:chloroform–isoamyl alcohol. The RNA was precipitated with three volumes of 95% ethanol. Transcription products were separated by electrophoresis on 10% acrylamide, 8 M urea gels and excised from the wet gels using an autoradiograph as a guide. RNA was eluted from the gel, precipitated with ethanol, dried, and resuspended in 250 μl of diethylpyrocarbonate-treated water. The sample was desalted by applying to a 10-ml Sephadex G-25 column. Peak fractions were identified by liquid scintillation counting, and the
samples were pooled and concentrated by centrifugation in a Speed-Vac concentrator (Savant Instruments). Internally labeled, high specific activity \(^{32}P\) UMP RNA (100–200 cpm/mmol) was synthesized for mobility shift and photo-cross-linking assays by including 1 mCi of \(^{32}P\)UTP per 1 ml of transcription reaction and lowering the concentration of UTP to 0.1 mM; all other ribonucleoside triphosphates were at the highest concentration. For the synthesis of both substrates, high specific activity RNA for guanylyltransferase assays and competition of ATPase and ATP cross-linking assays (37), 5 µCi of \(^{32}P\)UTP was included in the transcription reaction, thus facilitating the detection of RNA during purification.

**Enzyme Purification**

Synthesis and Purification of D1R Subdomains—Transfected BL21(DE3)plysS were induced with IPTG under low temperature conditions as described previously (23), except cells were induced with 200 µM IPTG in the presence of 2% ethanol. Cultures were shaked overnight at 15–18°C, after which the cells were harvested by centrifugation and stored at –70°C until needed.

D1Rcoo—The partial purification of D1R carboxy-terminal deletion mutants follows the purification scheme described for D1R-1–545 and as depicted in Fig. 3 (see “Results”) through the DEAE-cellulose step. BL21(DE3)plysS pET8c D1Rcoo cells harvested from 1-liter inductions were frozen and subsequently thawed in lysis buffer (2 mg of cells, 1 ml of NAL (3), adjusted to pH 7.4 and 10000 x g for 60 min at 4°C. Ammonium sulfate was added to 50% saturation, the precipitate collected by centrifugation, resuspended in 3 ml of buffer A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol (w/v), and 50 mM NaCl), and dialyzed against 4 liters of buffer A with one change of buffer. DEAE-cellulose (10 ml, Whatman DE52), preequilibrated with buffer A, was added to the ammonium sulfate dialyze, and the two components were mixed by gentle rocking at 4°C for 1.5 h. The DEAE-cellulose resin was pelleted by low speed centrifugation, and the supernatant fractions were assayed for protein composition by SDS-PAGE on 12.5% acrylamide gels and by Western blot analysis, using polyclonal antiserum raised against the polypeptide segment of the D1R protein. Enzyme activity RNA formation were assayed as described below. A supernatant fraction from BL21(DE3)plysS prepared in an identical manner was used as a control sample.

Purification of D1R-1–545—Fig. 3 depicts the scheme employed for the purification of D1R-1–545. The initial steps, from lysis through the 100 000 x g centrifugation step, were conducted as described for purification of the co-expressed capping enzyme subunits (23) with the following modifications. Typically 250 g of BL21(DE3)plysS pET8c D1R-1–545 were thawed in 500 ml of lysis buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (w/v), 10 mM β-mercaptoethanol, 50 mM NaCl, 0.1% sodium deoxycholic acid, 0.01% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. Following the preparation of the polyethyleneimine (PEI) supernatant and high speed centrifugation, ammonium sulfate was added to the S100 fraction to a final concentration of 60%, stirred for 30 min at 4°C, and centrifuged at 10 000 x g for 30 min at 4°C, and centrifuged at 100 000 x g for 60 min at 4°C. Ammonium sulfate was added to 50% saturation, the precipitate collected by centrifugation, resuspended in 3 ml of buffer A (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol (w/v), and 50 mM NaCl), and dialyzed against 4 liters of buffer A with one change of buffer. DEAE-cellulose (10 ml, Whatman DE52), preequilibrated with buffer A, was added to the ammonium sulfate dialyze, and the two components were mixed by gentle rocking at 4°C for 1.5 h. The DEAE-cellulose resin was pelleted by low speed centrifugation, and the supernatant fractions were assayed for protein composition by SDS-PAGE on 12.5% acrylamide gels and by Western blot analysis, using polyclonal antiserum raised against the polypeptide segment of the D1R protein. Enzyme activity RNA formation were assayed as described below. A supernatant fraction from BL21(DE3)plysS prepared in an identical manner was used as a control sample.

Enzyme Assays

**ATPase—**A standard assay contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl2, 10 mM β-mercaptoethanol, 5 mM ATP, 0.5–1 µCi of \(^{32}P\)ATP (DuPont NEN, 6000 Ci/mmol) and enzyme in a 20-µl reaction volume. Enzyme was added last to a prechilled reaction mixture, shifted to 37°C for 5 min, and quenched by adding EDTA to a final concentration of 50 mM. A 2-µl aliquot was spotted onto PEI-cellulose thin layer chromatography plates (Alltech) which were developed in 0.75 M LiCl, 1 M acetic acid, and 0.75 M acetic acid, and visualized by autoradiography, and the radio labeled spots were excised from the TLC plate and quantified by Cerenkov counting. The assay was linear up to 20 min at enzyme concentrations between 50 and 1000 nM. Enzyme activity was typically measured in the presence of all enzyme dilutions.

RNA 5′-Triphosphatase–A standard assay was similar to the radiochemical ATPase assay with the following differences: each assay contained 2 mM MgCl2 and 20 µM \(^{32}P\)-labeled RNA (>2000 cpm/mmol), in a 10-µl reaction volume. Reactions were incubated for 2 min at 37°C and quenched with 2.5 µl of 25 mM EDTA, and 2 µl were spotted onto PEI-cellulose TLC plates. Plates were developed in 0.75 M KH2PO4, pH 3.4 (13). The assay was linear for up to 5 min, at enzyme concentrations between 10 and 50 nM. A 10 nM enzyme concentration was typically used in kinetic analyses and the assay was quantified as described above for the ATPase assay. In competition experiments measuring the effect of different oligonucleotides on RNA 5′-triphosphatase activity, a 5 µM \(^{32}P\)-labeled 23-mer RNA substrate concentration was used. RNA 5′-triphosphatase activity was also measured in the presence of varying concentrations of oligonucleotides which included phosphorylated RNA, 5′-OH RNA (Oligos, Etc.), and single-stranded DNA, all 23 nucleotides in length and of the same sequence as the triphosphorylated RNA substrate (with uracil replaced by thymine in DNA).

**Guanylyltransferase—**The guanylylation of RNA comprises two half reactions: the formation of an enzyme–guanylyltransferase (PEI) complex, and the subsequent transfer of GMP to the 5′-terminal of phosphorylated RNA (the transguanylylation reaction) (7, 8). Measurement of E–GMP formation was as described elsewhere (27). The transguanylylation of RNA was measured in a standard reaction containing 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 2 mM MgCl2, 100 µM GTP, 2 µCi of \(^{32}P\)GTP (800 cpm/mmol), triphosphorylated RNA 23-mer, and 50 nM enzyme, in a 10-µl reaction volume. For RNA saturation experiments, the GTP concentration was held constant at 100 µM and RNA was varied up to 20 µg; for GTP saturation experiments, the RNA concentration was held constant at 5 µg, and the GTP concentration was varied up to 100 µM. Reactions were carried out at 37°C for 4 min and quenched with 10 µl of 2 × RNA sample buffer (80% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanid). The samples were heated for 5 min at 95°C and then loaded onto a 10% acrylamide gel (19:1 acrylamide:bisacrylamide) containing 8% urea. The gels were run at 150 volts in 1 × TBE buffer (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, 2.5 mM EDTA), fixed for 10 min in 40% methanol, dried, and visualized by autoradiography. \(^{32}P\)-Radio labeled bands corresponding to guanylylated RNA were excised and quantified by liquid scintillation counting.

**RNA Binding—**RNA binding was measured by a gel mobility shift assay. In this analysis, varying amounts of enzyme were preencubated with ice for 15 min in a 20-µl reaction containing 50 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 5% glycerol (w/v), and 20 µM of \[^{32}P\]JUMP RNA (200 cpm/mmol). The assay mixture was then loaded onto a native acrylamide gel (6% acrylamide with 19:1 acrylamide:bisacrylamide, 5% glycerol, 0.25 × TBE) prequilled at 4°C and run at 200 V. Gels
were dried and visualized by autoradiography. The bound RNA was determined either by quantifying the disappearance of free (unshifted) RNA or alternatively as a percentage of the input RNA determined by scintillation counting of both the bound and unbound species.

Results

DIr Carboxyl-terminal Deletion Analysis—Previous work (19, 21, 23, 24) has enabled a partial mapping of the catalytic domains for each of the mRNA cap formation activities. The RNA 5'-triphosphatase, ATPase, and guanylyltransferase activities reside in a 60-kDa amino-terminal subdomain of the large subunit, D1R. The remaining portion of the heterodimeric enzyme, comprised of the carboxyl terminus of D1R from amino acids 545-844 in addition to the entire small subunit (D12L), constitutes the methyltransferase domain (24, 40). In an attempt to define the minimal primary sequence of D1R required for phosphohydrolase activity (37) and to ascertain whether this activity could be separated from the guanylyltransferase activity, combinatory photo-cross-linking of RNA to the enzyme was achieved. The truncated D1R proteins were partially purified from bacterial lysates through a batch DEAE cellulose chromatography step. The DEAE-cellulose fractions (10 μg of total protein) were assayed for ATPase activity (B), guanylyltransferase activity, measured here by the formation of the E–GMP intermediate (C), and D1R specific protein levels by Western blot analysis (D). Each activity was measured relative to the control supernatant, C, prepared from BL21(DE3)plysS under identical conditions. A, map of D1R carboxyl-terminal deletion mutants, indicating the positions of their respective carboxyl termini. Also shown is lysine 260 of the guanylyltransferase active site, required for the formation of the E–GMP intermediate.

were dried and visualized by autoradiography. The bound RNA was determined either by quantifying the disappearance of free (unshifted) RNA or alternatively as a percentage of the input RNA determined by scintillation counting of both the bound and unbound species.

RNA Photo-cross-linking—The direct photo-cross-linking of RNA to enzyme was carried out as described for ATP (37) except 30-μl reactions included 1 μl [α-32P]UMP RNA 23-mer (~100 cpm/fmol) used as cross-linking reagent, 3 pmol of D1R1–545, and varying concentrations of either 5'-OH RNA or DNA 23-mer oligonucleotides. The samples were treated with 5 μg of RNase A for 30 min at 37 °C prior to adding 0.3 volume of 4 × SDS sample buffer. Typically, less than 4% cross-linking of RNA to the enzyme was achieved.

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Expression of D1R<sup>1-545</sup>—Based on the results in Fig. 2, the T7 expression plasmid pET8c D1R<sup>1-545</sup>. Fig. 1, was constructed to direct the synthesis of a truncated D1R protein that terminates at amino acid 545. BL21(D33)plysS D1R<sup>1-545</sup> cultures were induced with IPTG as described. The synthesis of an approximately 62-kDa D1R protein was confirmed by SDS-PAGE and Western immunoblot analysis. As was the case for the full-size capping enzyme, heterologously expressed D1R<sup>1-545</sup> and mRNA capping enzyme exhibited comparable substrate saturation kinetics for both RNA 5′-triphosphatase and GMP formation.

RNA Binding—Prior results (24, 36) have demonstrated that RNA binding to the mRNA capping enzyme is complex. Both RNA mobility shift analyses and UV photo-cross-linking studies indicate that at least two RNA binding sites exist within separate regions of the large (D1R) subunit corresponding to the NH<sub>2</sub>-terminal 60-kDa domain and the carboxyl terminus of D1R from amino acids 498–844 which contains the methyltransferase domain. Moreover, the rate of guanylylated RNA formation exhibited both RNA 5′-triphosphatase (Fig. 4A) and ATPase (Fig. 4B). To assess the relative guanylyltransferase activity, both half-reactions, the formation of the covalent E–GMP intermediate and the transguanylylation of 23-mer RNA, were measured. In comparison to the intact capping enzyme, D1R<sup>1-545</sup> formed E–GMP with the same stoichiometry (Fig. 5A) and was equally competent in transferring GMP to acceptor RNA (Fig. 5B). Moreover, the rate of guanylylated RNA formation exhibited the same RNA (Fig. 5C) and GTP (Fig. 5D) concentration dependences. A comparative kinetic summary for ATPase, RNA 5′-triphosphatase, and guanylyltransferase activities is presented in Table II. From these analyses, we conclude that the phosphohydrolase and guanylyltransferase kinetic activities in the D1R subdomain are not influenced by the absence of the methyltransferase domain.
D12L subunit was observed (24). To further characterize RNA binding to the D1R1–545 subdomain and to compare its behavior to the methyltransferase domain, we again employed both RNA mobility shift analyses and direct UV photo-cross-linkage of RNA to D1R1–545, the methyltransferase domain, or the intact capping enzyme.

Mobility Shift Assays—Binding of 23-mer RNA to the intact capping enzyme yields two products (Fig. 6A), a faster migrating species at low enzyme concentrations and an electrophoretically retarded complex at high enzyme levels. The appearance of a slower migrating RNA-protein complex at higher enzyme concentrations concomitant with a reciprocal disappearance of the faster migrating species is consistent with up to two molecules of enzyme bound per 23-mer RNA. Similar results were also obtained with the methyltransferase domain, Fig. 6B. The S0.5 observed for the holoenzyme and the methyltransferase domain of 35 nm and 95 nm, respectively (Table III), demonstrate similar RNA binding affinities. D1R1–545 also exhibits two shifted RNA-protein complexes with the slower electrophoretic species predominating at higher levels of D1R1–545, Fig. 6C. In comparison to the holoenzyme, however, the D1R1–545 subdomain binds triphosphorylated 23-mer RNA with an approximately 20-fold weaker affinity (720 nm), Fig. 6D. Taken collectively, these data demonstrate that the methyltransferase domain accounts for a major component of total RNA binding in the intact capping enzyme.

Identical RNA binding saturation results were also obtained when RNA of a different length (e.g. a 60-mer) or RNA possessing a diphosphorylated 5' end was employed (data not shown). This fact held true for both the D1R1–545 and the methyltransferase domains. These results indicate that maximal RNA binding is achieved at modest chain length of 23 nucleotides and that this binding does not require the terminal 5'γ-phosphate, at least when measured under the conditions of this assay. This observation agrees with those of Luo and Shuman (36), indicating capped RNA, triphosphorylated RNA, and RNA lacking a phosphorylated 5' end all interacted with the intact capping enzyme in a similar manner.

RNA UV Photo-cross-linking—RNA binding was investigated further by directly UV cross-linking radiolabeled RNA to D1R1–545. In a preliminary analysis, D1R1–545 cross-linked RNA with an approximately 2% efficiency at saturating RNA concentrations (1–2 μM). The specificity of RNA cross-linking for binding at the RNA 5'-triphosphatase active site in D1R1–545 was tested by measuring the degree of inhibition of both RNA cross-linking and RNA 5'-triphosphatase activity in the presence of competing oligonucleotides, Fig. 7. The competing single-stranded DNA and RNA 23-mers possessed the same sequence as the radiolabeled RNA, but lacked a triphosphorylated 5' terminus. In these experiments, cross-linked samples were treated with RNase A following UV irradiation, both to remove RNA not covalently associated with the enzyme and to hydrolyze susceptible regions of RNA bound to the protein, thus permitting the migration of the modified protein as a single component during gel electrophoresis. Omission of RNase treatment resulted in a diffuse radiolabeled band (Fig. 7A, lane 1). 5'-OH RNA inhibited RNA cross-linking by 50% at approximately 15 μM while 23-mer DNA oligonucleotide demonstrated only weak inhibition of RNA cross-linking to D1R1–545 (Fig. 7B). These data correlate with the relative ability of the same oligonucleotides to inhibit RNA 5'-triphosphatase activity (Fig. 7C). In this analysis, diphosphorylated 23-mer RNA exhibited 50% inhibition at less than 10 μM, similar to the degree of inhibition observed when cold triphosphorylated RNA was included in the assay to demonstrate a substrate dilution effect. These results indicate that the

![Graph](image_url)

**Fig. 5.** Comparison of D1R1–545 and holoenzyme guanylyltransferase kinetics. Guanylyltransferase kinetics, including both E–GMP formation (A) and the transguanylylation of 23-mer RNA (B–D) were determined for D1R1–545 (C) and the full-sized mRNA capping enzyme (D). A, E–GMP reactions were carried out for 60 s on ice in the presence of varying amounts of enzyme, quenched with 2 × SDS sample buffer, boiled for 5 min, and the covalently modified enzyme separated by SDS-PAGE on 10% acrylamide gels. The stoichiometry of E–GMP formation was determined by excising the radiolabeled protein from the dried gel and quantifying by Cerenkov counting. B, 5 μM triphosphorylated 23-mer RNA was incubated with varying concentrations of enzyme in the presence of 100 μM GTP and 2 μCi [γ-32P]GTP (800 Ci/mmol) in a 10 μl reaction volume. Reactions were carried out for 4 min at 37 °C, quenched with 2 × RNA sample buffer and the guanylylated RNA resolved by electrophoresis on 10% polyacrylamide gels containing 8 M urea. Mols of guanylylated RNA were determined by Cerenkov counting of radiolabeled RNA excised from the dried gel. C and D, comparative guanylyltransferase substrate saturation kinetics. Guanylyltransferase velocities were measured as described in B except either the RNA concentration was varied and the concentration of GTP held constant at 100 μM (C) or the GTP concentration was varied and the RNA concentration held constant at 5 μM (D).

**TABLE II**

Summary of kinetic comparison of D1R1–545 to holoenzyme

| Substrate | ATPase | RNA 5’-triphosphatase | Guanylyltransferase |
|-----------|--------|----------------------|---------------------|
|            | K<sub>m</sub> | Turnover no. | K<sub>m</sub> | Turnover no. | K<sub>m</sub> | Turnover no. |
|            | μM | min<sup>−1</sup> | μM | min<sup>−1</sup> | μM | min<sup>−1</sup> |
| Capping enzyme | 840 | 384 | .80 | 30 | 13.1 | 1.4 |
| D1R<sup>1–545</sup> | 1040 | 500 | .90 | 46 | 15.5 | 1.6 |

Substrate saturation kinetics were measured for ATPase, RNA 5’-triphosphatase, or guanylyltransferase, comparing D1R1–545 with the full-size capping enzyme. Kinetic values were determined from a linear transformation (Lineweaver-Burke) of the data presented in Figs. 4 and 5.
The diphosphorylated RNA product binds to D1R1–545 with an affinity similar to the triphosphorylated substrate. In contrast, 5'-OH RNA inhibited RNA 5'-triphosphatase activity less effectively, with 50% inhibition occurring at 40 μM, indicating that RNA lacking a phosphorylated terminus does not bind as well to D1R1–545 as either tri- or diphosphorylated RNA. Single-stranded DNA did not effectively inhibit RNA 5'-triphosphatase activity, demonstrating that the observed binding is restricted to RNA. Although these oligonucleotides inhibited RNA photo-linkage and RNA 5'-triphosphatase activity to different extents, the relative pattern of inhibition by 5'-OH RNA and single-stranded DNA was consistent in the two experiments. This observation indicates that the RNA binding to D1R1–545, measured here by UV photo-linkage, is catalytically productive in RNA 5'-triphosphatase activity.

**DISCUSSION**

The vaccinia virus mRNA capping enzyme catalyzes three sequential reactions in the mRNA cap formation pathway (7-...
Knowledge of the number of active sites catalyzing these reactions and their physical arrangement is fundamental to our understanding of the mechanism of cap formation. Any model which addresses this issue must be consistent with the known domain structure of the enzyme. The mRNA capping enzyme is comprised of two subdomains (19–21, 23), a 60-kDa amino-terminal domain of the large D1R subunit which possesses the RNA 5′-triphosphatase, guanylyltransferase, and nucleoside triphosphate phosphohydrolase active sites (20, 23), and a separate heterodimeric domain comprised of the carboxy-terminal portion of D1R from amino acids 545–844 together with the small (D12L) subunit, possessing the (guanine-7)-methyltransferase active site (23, 24, 40). Therefore, a simple model predicting a single active site for concerted catalysis is impossible; at least two active sites must exist.

We favor a model depicting three separate active sites, one for each step in the cap formation pathway. The three site model requires that the RNA 5′-triphosphatase and guanylyltransferase activities are catalyzed at separate locations, raising the possibility that the first two active sites may reside in two independent domains. As a first test of this hypothesis, carboxy-terminal deletions in the D1R gene were constructed, expressed in E. coli, and ATPase and guanylyltransferase activities measured. The results of these measurements confirm that the D1R 60K subdomain is the minimal functional unit for both activities and delimit the carboxy terminus of this active domain to between amino acids 520 and 545. The failure of this approach to generate deletion mutants exhibiting only ATPase or guanylyltransferase activity may indicate a close conformational linkage of the phosphohydrolase and guanylyltransferase active sites. In a related experiment, we observed that the amino-terminal deletion mutant D1R1–524–545 lacked any measurable ATPase activity or the ability to form the covalent E–GMP intermediate (data not shown). These results further support the assignment of the ATPase/guanylyltransferase domain structure to include amino acids 1 to approximately 545. Although the physical separation of the two active sites has not yet been achieved, a kinetic argument for separate RNA 5′-triphosphatase and guanylyltransferase active sites can be made (37).

The assignment of the RNA 5′-triphosphatase and guanylyltransferase activities to the D1R1–545 subdomain potentially simplifies further structure-function analyses. In order to purify enough of this domain, a truncated D1R protein with a defined carboxy terminus at amino acid 545 was cloned and expressed in E. coli. Purification of soluble enzyme was achieved by large-scale induction of B21(D3)plysS D1R1–545 under low temperature conditions followed by sequential chromatographic fractionation of the lysate. The purification of D1R1–545 builds on the protocol for the purification of the full-sized capping enzyme. Unlike the intact enzyme, however, D1R1–545 binds poorly to phosphocellulose or hydroxyapatite. This property mimics the chromatographic behavior of the D1R 60-kDa domain proteolysis product observed during the purification of the co-expressed subunits (20, 23). As expected, ATPase and E–GMP activities co-eluted with the D1R protein throughout purification. A yield of 25 mg of soluble D1R protein was typically achieved from the lysis of 250 g of cells.

A kinetic comparison of the D1R1–545 subdomain to the full-sized capping enzyme demonstrated that the D1R1–545 domain possessed comparable ATPase and RNA 5′-triphosphatase substrate saturation kinetics. Likewise, this subdomain was fully comparable to the holoenzyme in guanylyltransferase kinetics, both in the stoichiometry of E–GMP formation and in the transguanylylation of RNA. Since the kinetic behavior of D1R1–545 is equivalent to the intact capping enzyme, the presence of the methyltransferase domain in the holoenzyme must not influence the turnover of RNA at either the RNA 5′-triphosphatase or guanylyltransferase active site. A corollary to this fact states that the structural elements which specify RNA binding for the RNA 5′-triphosphatase and guanylyltransferase active sites must reside within D1R1–545.

To investigate the relationship of RNA binding to capping enzyme function, we employed mobility shift and UV photo-cross-linking analyses as measures of RNA binding to both subdomains and to the full-size capping enzyme. Each subdomain of the mRNA capping enzyme binds to 23-mer triphosphorylated RNA in a manner indicating that up to two molecules of enzyme bind per RNA molecule, at high enzyme concentrations. The affinity of the methyltransferase domain for RNA is nearly 8-fold greater than D1R1–545 and 3-fold weaker than the intact capping enzyme. This result indicates that the methyltransferase domain possesses the major RNA binding component of the holoenzyme.

Although the binding of triphosphorylated RNA to the D1R1–545 subdomain is apparently weaker than binding to the methyltransferase domain, it represents catalytically productive binding at the RNA 5′-triphosphatase active center. This conclusion is based on the fact that photo-linkage of triphosphorylated RNA is inhibited by 5′-OH RNA and to a much lesser extent, single-stranded DNA in a concentration dependent manner, consistent with the relative inhibition of RNA 5′-triphosphatase activity by these same oligonucleotides. The relationship of this inhibition to the binding of RNA at the guanylyltransferase active site is not addressed per se by these experiments. We cannot differentiate between separate RNA binding sites for each active site or a single site for the binding of diphosphorylated RNA shared by the two catalytic sites.

In considering these data in the fuller context of a single round of RNA cap formation, it is surprising that that the binding of RNA to the methyltransferase domain is roughly 8-fold stronger than binding to D1R1–545, yet the presence of this domain exerts no influence on the RNA substrate saturation kinetics for either the RNA 5′-triphosphatase or guanylyltransferase activities. This fact suggests that the binding of RNA to either active site in D1R1–545 must not be a rate-limiting step in the reaction sequence. Alternately, RNA binding at the methyltransferase active site is unrelated to binding at either catalytic site in the D1R1–545 subdomain and perhaps reflects a binding function separate from capping at the 5′ terminus.

A weak but specific association of RNA with D1R1–545 does fit with the three active site model for mRNA cap formation. In this model, the RNA 5′-terminus is processed sequentially at each site and in a fashion which is kinetically autonomous from the next step. It is sensible that the nascent RNA binds to the first active site (i.e., the RNA 5′-triphosphatase active site) with the greatest specificity but not necessarily the highest affinity. Subsequent to catalysis, the product must move to the next active site; a site exhibiting less specificity for the 5′-triphosphorylated RNA but a greater affinity for RNA would promote this transfer.

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