Intrinsic RNA (Guanine-7) Methyltransferase Activity of the Vaccinia Virus Capping Enzyme D1 Subunit Is Stimulated by the D12 Subunit

IDENTIFICATION OF AMINO ACID RESIDUES IN THE D1 PROTEIN REQUIRED FOR SUBUNIT ASSOCIATION AND METHYL GROUP TRANSFER*

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Vaccinia virus mRNA capping enzyme, a heterodimer of virus-encoded D1 and D12 subunits, catalyzes three steps in the synthesis of the m7GpppN cap. By expressing portions of the subunits in bacteria, singly and together, we have localized the RNA (guanine-7) methyltransferase domain to a 305-amino acid carboxy-terminal segment of the D1 polypeptide (residues 540-844) complexed with the D12 protein. We find that the purified carboxyl D1 protein has a weak intrinsic methyltransferase activity, indicating that the catalytic center resides within this subunit. The basal level of activity can be stimulated 100-fold by addition of purified D12 protein, which is itself catalytically inert. The carboxyl region of D1 forms a heterodimer with the D12 subunit in vitro and in vivo. Analysis of alanine substitution mutants of the D1 protein identifies amino acid residues important for subunit interaction. Our results suggest that subunit heterodimerization is necessary, but not sufficient, for full methyltransferase activity. A mutation of vicinal positions His-682-Tyr-683 that specifically affects catalytic activity but not subunit interaction implicates these residues as constituents of the active site.

mRNA capping occurs by a series of three enzymatic reactions in which the 5'-triphosphate terminus of a primary transcript is first cleaved to a diphasphate-terminated RNA by RNA triphosphatase, then capped with GMP by RNA guanylyltransferase, and finally methylated at the N-7 position of guanine by RNA (guanine-7) methyltransferase (1). All three steps in cap formation are catalyzed by the vaccinia virus mRNA capping enzyme, a heterodimeric protein containing virus-encoded polypeptides of 95 and 33 kDa (2, 3). The vaccinia capping enzyme has been purified to homogeneity, and the viral genes encoding the two subunits have been identified.

The guanylyltransferase reaction has been dissected in some detail. Transfer of GMP from GTP to the 5'-diphosphate terminus of RNA occurs in a two-stage reaction involving a covalent enzyme-GMP intermediate (4). Both steps are readily reversible.

\[
\begin{align*}
(i) \quad E + pppG &\rightarrow EpG + PP \\
(ii) \quad EpG + p\text{RNA} &\rightarrow G\text{pppRNA} + E
\end{align*}
\]

(Eq. 1)

The methyltransferase reaction proceeds if the D1 subunit is present as a heterodimer with the D12 subunit. Methyltransferase activity implicates two conserved amino acid residues in the D1 subunit, His-682 and Tyr-683, in the formation of a covalent intermediate with the D12 subunit. The present study addresses the contribution of individual enzyme subunits to the methyltransferase domain. We now find that the D1 subunit of the capping enzyme has a weak intrinsic methyltransferase activity that is markedly stimulated by the D12 subunit. By expressing truncated versions of the D1 gene, we localize the methyltransferase component of the D1 enzyme subunit to a carboxy-terminal segment from amino acids 498-844. This region of the D1 polypeptide is capable of forming a heterodimer with the D12 subunit in vitro. Mutational analysis of the D1 subunit identifies residues essential for subunit interaction and transmethylation.

EXPERIMENTAL PROCEDURES

T7-based Plasmids for Expression of His-tagged Capping Enzyme Subunits—Plasmid pET-His-D1(498-844) (Fig. 1A) was constructed by excising the D1(498-844) coding sequence from pET-C-D1 (described in Ref. 10) by digestion with NdeI and HindIII and inserting it at the NdeI and HindIII sites of pET14b (Novagen), thereby placing it in-frame with the resulting plasmid, each gene is driven by a separate, tandemly oriented T7 promoter (Fig. 1C). The truncated allele D1(540-844) was created by site-directed mutagenesis as follows. A BglII-HindIII fragment containing the D1(498-844) expression cassette was transferred to the phagemid plasmid pBS— (Stratagene). This plasmid, pBS-His-D1(498-844), was transformed into Escherichia coli C600. Uracil-substituted single-
stranded DNA was prepared for use as a template for oligonucleotide-directed mutagenesis (11). An oligonucleotide DNA primer was designed to create a Gin to Met coding substitution at residue 540 and to introduce a new NdeI restriction site at this position. After confirming the mutation by dyeodeoxynucleotide sequencing of plasmid isolates, a fragment containing the D1 coding sequence from amino acids 540-844 was engineered into NdeI and HindIII and transferred to pET-His-D1(540-844) (Fig. 1D). An allele of D1(540-844) lacking the His tag was also constructed by inserting the same fragment to pET3c to yield pET-D1(540-844). The His-D1(540-844) cassette was transferred to pET-D12 to yield the coexpression plasmid pET-D12/His-D1(540-844) (Fig. 1E). A coexpression plasmid containing the His tag on the D12 protein was constructed by subclonin the D1(540-844) cassette into pET-His-D12 to produce the construct shown in Fig. 1F. The various expression plasmids were transformed into E. coli BL21(D3E).2

Expression and Purification—E. coli BL21(DE3)/pET transformants were inoculated into LB medium containing 0.1 mg/ml ampicillin and grown at 37 °C until the A600 reached approximately 0.6. Cultures (200-ml cultures in 300-ml flasks) of cells bearing the single expression plasmids pET-His-D12 and pET plasmids coexpressing both subunits; 600-ml cultures of cells bearing the single expression plasmids pET-His-D1(498-844) and pET-His-D1(540-844)) were then plated on 20% sucrose, 10% glycerol gradient containing 0.2 M NaCl in buffer A. Gradients were prepared by centrifugation, and pellets were stored at -80 °C. All subsequent procedures were performed at 4 °C. Cell lysis was achieved by treatment of thawed, resuspended cells with lysozyme and Triton X-100 as described, using modified lysis buffer containing 10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 10% sucrose (12). Insoluble material was removed by centrifugation at 18,000 rpm for 30 min in a Sorvall SS34 rotor. The supernatants were mixed with 1 ml of Ni-NTA-agarose resin (Qiagen) for 1 h. The slurry was poured into a column and then washed with lysis buffer. The columns were eluted stepwise with IMAC buffer (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 1 mM phenylmethylsulfonfyl fluoride, 10% glycerol containing 5, 25, 45, 65, and 100 mM imidazole). The polypeptide composition of the column fractions was monitored by SDS-PAGE. Elute fractions enriched for the expressed capping enzyme subunits (typically, the subunits were eluted at 45 and 65 mM imidazole) were pooled and then mixed against buffer B (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 1 mM diethiothreitol, 0.05% Triton X-100, 5% glycerol). Each dialyse was applied to a 1-2-ml column of phosphocellulose that had been equilibrated with buffer B. The column was eluted stepwise with buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM diethiothreitol, 0.1% Triton X-100, 10% glycerol) containing 0.5, 0.1, 0.2, 0.5, and 1 mM NaCl step. Coexpressed subunit heterodimers were recovered in both 0.2 and 0.5 M NaCl fractions. The yield of capping enzyme subunits at the phosphocellulose step was typically 50% (Hедин and Holmgren, unpublished observations). The yield of capping enzyme subunits at the phosphocellulose step was about 40% (Hedin and Holmgren, unpublished observations). The yield of capping enzyme subunits at the phosphocellulose step was about 40% (Hedin and Holmgren, unpublished observations). The yield of capping enzyme subunits at the phosphocellulose step was about 40% (Hedin and Holmgren, unpublished observations).

The capping enzyme subunits were expressed in E. coli using a T7-based expression-cassette system, and in Fig. 6. NdeI-HindIII fragments containing the various D1-Ala alleles were subcloned back into pET14b. pET-His-D1-Ala plasmids or pBS-His-D1-Ala plasmids were linearized downstream of the expression cassette prior to use as templates for transcription in vitro by T7 RNA polymerase.

Transcription and Translation in Vitro—Capped synthetic mRNAs encoding the His-D1(498-844) and D12 subunits, or Ala-mutated derivatives thereof, were generated by transcription of the appropriate plasmids by T7 RNA polymerase in the presence of cap analog m'GpppA.2C (Stratagene) according to the vendor’s instructions. Integrity of each transcript was confirmed by electrophoresis of the transcription reaction products through a formaldehyde-containing 1% agarose gel and visualizing of the RNA by staining with ethidium bromide. RNA transcripts were translated (in vitro) for 15 min in rabbit reticulocyte lysate. Translation reactions were constituted using an in vitro translation kit (Promega) according to the manufacturer’s instructions. The reactions were incubated at 37 °C for 60 min in the presence of 0.2 Ci/ml [35S]methionine (>800 Ci/mmol). mRNA was included in each reaction at a concentration of 30 μg/ml. In those reactions where translation products were not radiolabeled, 1-methionine was included at 0.11 mM concentration. Unlabeled translation products were separated from low molecular weight material by gel filtration. Aliquots (60 μl) of translation reactions were applied to 1-ml spin columns of Sephadex G-25 that had been equilibrated with 0.1 mM NaCl in buffer A. Each translation reaction was then centrifuged at 4 °C in a clinical centrifuge. Immuno precipitation—An aliquot of the in vitro translation reaction mixture (4 μl of reticulocyte lysate) was diluted in 100 μl of immunoprecipitation buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM MgCl2, 0.1% Nonidet P-40, 0.02% sodium azide) and incubated on ice for 20 min. Insoluble material was removed by microcentrifugation for 10 min. The supernatant was collected and supplemented with 1 μl of rabbit anti-D1(498-844) antisera (10). This mixture was then incubated overnight at 4 °C with gentle rotation. Insoluble material was removed by microcentrifugation for 10 min. The supernatant was collected and resuspended with 50 μl of reticulocyte lysate. This reaction mixture was then incubated for 30 min at 4 °C with gentle rotation. The Sepharose beads were collected by microcentrifugation and washed four times with 0.75 ml of immunoprecipitation buffer. Bound proteins were eluted from the beads in 40 μl of sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue). This material was electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. Radiolabeled polypeptides were visualized by autoradiographic exposure of the dried gel.

RESULTS

Expression and Purification of His-tagged Capping Enzyme Subunits—Previous studies showed that the carboxyl-terminal region of the capping enzyme large subunit, D1(498-844), was capable of heterodimerization with the D12 subunit and that this heterodimer complex constituted an autonomous methytransferase domain (10). To examine the contribution of each domain to enzyme activity, attempts were made to express the D1(498-844) and D12 proteins in bacteria. Although it was initially shown that the full-length D1 and D12 proteins could be expressed in E. coli using a T7-based system, the level of soluble protein obtained from cells grown at 37 °C was generally low (7, 8, 14). This was especially so when the D1 or D12 subunits were expressed alone. To improve purification of the D1 and D12 proteins from bacterial lysates, we

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; AdoMet, S-adenosylmethionine.
Methyltransferase Domain of mRNA Capping Enzyme

Fig. 1. T7-based plasmids for the expression of vaccinia capping enzyme subunits. Construction of plasmids is as described under "Experimental Procedures." The figure depicts the salient functional elements of the expression cassettes. The complete coding sequence for the capping enzyme small subunit is denoted by D12. The coding sequence of the large subunit is indicated by the T7 promoter and histidine tag. The transcriptional control signals and amino-terminal histidine-rich leader peptides are arrayed as indicated by the boxed symbols. The direction of transcription from the T7 promoter is indicated by the arrows.

have constructed a set of T7-based expression vectors (pET-His-D1(498–844) and pET-His-D12) that add a short histidine-rich amino-terminal segment to otherwise intact coding regions (Fig. 1, A and B). A separate plasmid, pET-D12/His-D1(498–844), was engineered to allow coexpression of the His-tagged D1 subunit plus the native D12 subunit (Fig. 1C). The level of protein expression and protein solubility was improved by growing the bacteria containing these plasmids at 20°C with isopropyl-1-thio-β-D-galactopyranoside induction, as suggested by Higman et al. (8). This maneuver was useful for the D1/D12 coexpression constructs and for expression of D12 alone. However, the expression of D1 constructs alone was still plagued by low yield of soluble protein. The His tag allowed for rapid enrichment of the tagged protein based on the affinity of the tag for immobilized nickel. The tagged protein was eluted from the nickel column with imidazole and then purified further by phosphocellulose chromatography and glycerol gradient sedimentation.

SDS-PAGE analysis of the phosphocellulose fractions indicated that the preparations were virtually homogeneous with respect to the capping enzyme subunits (Fig. 2). The His-D1(498–844) and His-D12 preparations contained single polypeptides of 44 and 35 kDa (Fig. 2, lanes A and B). Coexpression of His-D1(498–844) and native D12 resulted in recovery of both polypeptides in seemingly equimolar amounts (lane C). As expected, the native D12 polypeptide migrated more rapidly during SDS-PAGE (33 kDa) than did the His-D12 protein (compare lanes B and C). Note that because only the D1 subunit was His-tagged in this case, the presence of the D12 subunit implied that subunit association had occurred in E. coli. The coexpressed His-D1(498–844) and D12 subunits cosedimented exactly during glycerol gradient sedimentation (not shown); SDS-PAGE analysis of the peak fraction showed an equivalent amount of each polypeptide (Fig. 2, glycerol gradient, lane C). The apparent sedimentation coefficient of 4.5 S of the His-D1(498–844)/D12 enzyme indicated that the purified protein was a heterodimer. In contrast, the sedimentation constants of the singly expressed proteins His-D12 (2.8 S) and His-D1(498–844) (3.3 S) were consistent with those of monomeric proteins.

Fig. 2. Purification of capping enzyme subunits expressed in bacteria. The polypeptide composition of the peak phosphocellulose and glycerol gradient protein fractions was assessed by SDS-PAGE. Protein preparations were derived from soluble lysates of bacteria bearing single expression or coexpression plasmids specified by the letter code shown in Fig. 1; the source of the expressed subunit fraction is indicated by letter above each gel lane. Equivalent amounts of the phosphocellulose (1 µg) and glycerol gradient (1 µg) preparations were applied to the 10% polyacrylamide gel. Polypeptides were visualized by staining with Coomassie Blue. The positions and sizes (kDa) of coelectrophoresed marker polypeptides is indicated at the right.

His-D1(498–844) (3.0 S) indicated that the individual subunits were monomeric. A minor 38-kDa polypeptide was detected in the glycerol gradient preparation of the His-D1(498–844) protein that was absent in the phosphocellulose fraction (Fig. 2, glycerol gradient, lane A); this species likely arose from proteolysis of His-D1(498–844) during sedimentation and handling of fractions. Previous studies showed that the D1 protein expressed in bacteria was extremely sensitive to proteolysis in the absence of the D12 subunit (7, 8). The His-D12 protein was homogeneous after glycerol gradient sedimentation (Fig. 2, glycerol, lane B).

Methyltransferase Activity of His-D1(498–844)/D12 Heterodimer—In agreement with earlier studies of the non-tagged heterodimer (10), the purified His-D1(498–844)/D12 heterodimer was active in methyl transfer (Fig. 3A). The methyltransferase assay, which is sensitive and specific, measures the conversion of 32P cap-labeled poly(A) to methylated cap-labeled poly(A) in the presence of unlabeled AdoMet. Reaction products are digested to cap dinucleotides with nuclease P1 and then analyzed by polyethyleneimine-cellulose thin layer chromatography, which resolves the GpppA cap from the methylated cap m’GpppA. The methyltransferase activity profile corresponded exactly to that of the His-D1(498–844) and D12 polypeptides during glycerol gradient sedimentation (not shown). The extent of cap methylation by the glycerol gradient fraction was proportional to the amount of input enzyme, and quantitative methylation was readily achieved (Fig. 3A). The preparation acted catalytically insofar as the yield of methylated cap was in excess of the molar amount of protein added. Assuming all proteins were active, we estimated a turnover number of 9 mol of cap methylation/mol of enzyme/min.

Intrinsic Methyltransferase Activity of the D1 Enzyme Subunit—We were able to demonstrate that the His-D1(498–844) subunit alone had associated (guanine-7) methyltransferase activity, albeit at a very low level, i.e. 0.1% the specific activity of the His-D1(498–844)/D12 heterodimer (Fig. 3B). Methyltransferase activity cosedimented with the His-D1(498–844) polypeptide during glycerol gradient sedimentation (not shown). Moreover, cap methylation by His-D1(498–844) was completely dependent on exogenous AdoMet (not shown). Assays of the purified small subunit, His-D12, indicated that less than 1% of the capped RNA substrate was methylated at all
levels of protein tested, up to 12 pmol of input His-D12.

Reconstitution of Methyltransferase from Purified Subunits—The feeble intrinsic methyltransferase activity of the purified His-D1(498–844) protein could be stimulated >100-fold by addition of purified His-tagged D12 subunit (Fig. 4A). Reconstitution of the native methyltransferase was achieved by simply coincubating the purified subunits in equimolar amounts (30 nM concentration of each) prior to titration of the mixture in standard methyltransferase assays. Increasing the amount of added His-D12 by a factor of 10 has only a modest impact (2-fold increase) in the specific methyltransferase activity of the subunit mixture. The turnover number of the reconstituted enzyme (1.6/min) was near that of the heterodimer purified from bacteria coexpressing the subunits.

Amino Truncation of the D1 Subunit—The experiments presented above show that the catalytic center of methyltransferase resides in the carboxyl portion of the large subunit and is subject to profound activation by the small subunit, a process that presumably requires the formation of a heterodimeric complex between the two. To refine the limits of the catalytic domain of the D1 protein, we truncated the D1(498–844) coding sequence by 42 amino acids from the amino terminus to create T7-based expression plasmids encoding the D1 residues 540–844. This segment was expressed alone as a His-tagged derivative, His-D1(540–844) (Fig. 1D), coexpressed in His-tagged form with native D12 (Fig. 1E) or coexpressed in native form with a His-tagged version of D12 (Fig. 1F). The subunits were purified from bacterial lysates by sequential nickel affinity and phosphocellulose chromatography steps. The proteins were nearly homogenous with respect to the capping enzyme subunits at the phosphocellulose step (Fig. 2). The 41-kDa His-D1(540–844) migrated more rapidly during SDS-PAGE than the His-D1(498–844) protein, as expected (Fig. 2, compare lanes A and D). The equivalent levels of His-D1(540–844) and D12 recovered from coexpression extracts indicated that heterodimerization had occurred in vivo (Fig. 2, lane E); similar results for the D1(540–844)/His-D12 combination attested that the His tag did not alter the subunit interaction (lane F). The coexpressed D1(540–844) and D12 proteins cosedimented during glycerol gradient sedimentation at 4.4 S, confirming that the proteins formed a heterodimer, regardless of which subunit was tagged (Fig. 2, glycerol, lanes E and F). His-D1(540–844) by itself sedimented as a monomer (3.0 S) (Fig. 2, glycerol, lane D and data not shown). The His-D1(540–844), like His-D1(498–844), was susceptible to partial proteolysis during the sedimentation step (note the minor 35-kDa species in lane D).

The heterodimeric enzymes His-D1(540–844)/D12 and His-D12/D1(540–844) were both active in cap methylation. The turnover numbers of these two enzymes (4–6/min) were similar to that of the His-D1(498–844)/D12 heterodimer. The His-D1(540–844) protein alone displayed a low level of intrinsic methyltransferase (Fig. 5B). The turnover number of His-D1(540–844), 0.02/min, was slightly higher than that of the His-D1(498–844) protein (Fig. 4, compare panels A and B). The methyltransferase activity of His-D1(540–844), like that of the longer version, could be stimulated 70-fold by coinubcation with purified His-D12 (Fig. 5B). These results show that the D1(540–844) segment possesses intrinsic methyltransferase activity, is capable of heterodimerizing with D12, and can respond to this interaction with enhancement of catalysis. A more extensively deleted allele, His-D1(579–844), when expressed in bacteria, yielded protein with no detectable methyltransferase activity alone or in response to purified His-D12 (not shown).

Mutational Analysis of the D1 Component of Cap Methyltransferase—Our initial efforts to map the methyltransferase domain by mutagenesis were guided by the alignment of the carboxyl regions of the vaccinia virus D1 polypeptide with related polypeptides encoded by Shope fibroma virus (15) and African swine fever virus (16) (Fig. 6). Nine mutated alleles of pET-His-D1(498–844) were created that contained single or clustered alanine substitutions at amino acid residues conserved between all three viral proteins (indicated by asterisks in Fig. 6). Alamine cluster mutagenesis entailed simultaneous substitution of 2 or 3 nearby residues with alanine. 7 mutants of this type were made (see Fig. 6). Two other mutants contained single alanine substitutions. The alanine-scanning mutagenesis strategy is well suited to the analysis of proteins whose structure-activity relationships are unknown. Alanine substitution eliminates the side chain beyond the beta-carbon, yet usually does not alter the main chain conformation or impose drastic electrostatic or steric effects (18–20).

Screening of the mutated D1(498–844) proteins for interaction with the D12 subunit and for methyltransferase activity was performed after expression of the protein in vitro trans-
Alicuots of the translation mixtures were adsorbed to protein A beads in the presence of polyclonal rabbit antiserum directed against the Dl(498–844) polypeptide (10). The His-Dl(498–844) singly translated was immunoprecipitated by this antisem (Fig. 7, lanes A10, B11, and C6), whereas the D12 polypeptide was not (lanes A15 and C8). When the two wild-type subunits were cotranslated, both labeled polypeptides were precipitated by anti-Dl serum (Fig. 7, lanes A11 and B12). Heterodimerization between the translation products is thereby inferred. Three of the nine mutated versions of His-Dl(498–844) were capable of binding to the cotranslated D12 subunit; these were K643A (lane A12), E736A (lane A13), and H682A,Y683A (lane C7). Four other mutant D1 proteins were apparently unable to bind cotranslated D12. These were L516A,V517A (lane B10), G569A,P564A,G566A (lane B14), D599A,G600A,G602A (lane B15), and G704A,G705A,V707A (lane B16). The mutant polypeptide D620A,D622A was partially active in binding to the D12 subunit (lane A14). These results establish a qualitative hierarchy of mutational effects on subunit-subunit interaction.

Effects of these mutations on methyltransferase activity of the cotranslated subunits was examined. Cong and Shuman (10) showed that active methyltransferase can be reconstituted in vitro by cotranslation of the large and small enzyme subunits. Incubation of gel-filtered reticulocyte lysates containing wild-type His-Dl(498–844) and D12 in vitro translation products with cap-labeled RNA substrate in the presence of AdoMet resulted in methylation of the substrate, seen as the release of m7GpppA after nuclease P1 digestion (Fig. 8, lane W7). Methyltransferase activity of the in vitro translation products required addition of AdoMet (not shown). Cap methylation activity was absent in mock-translated reticulocyte lysate or when the D12 subunit was translated alone (Ref. 10 and data not shown). Thus, our experiments concerning the functional consequences of D1 mutations apply only to the “native” methyltransferase activity manifest in the presence of D12.

Of the nine mutated D1 polypeptides, only two (K643A and E736A) had significant methyltransferase activity (Fig. 8). Note that these two proteins retained the ability to heterodimerize with D12. Of the remaining seven mutants that were cotranslated with D12, six displayed no methyltransferase activity in this assay, even when the autoradiogram in Fig. 8 was subjected to prolonged exposure. A mere trace level of cap methylation was seen for the G704A,G705A,V707A protein cotranslated with D12 upon prolonged exposure (not shown).

It is noteworthy that five of the seven D1(498–844) mutants that were grossly defective for methyltransferase activity in the presence of D12 were also defective in binding to D12. This indicated that subunit heterodimerization was essential to achieve native levels of enzyme activity. This was consistent with previous studies of deletion mutants of the D1 and D12 polypeptides in which heterodimerization and methyltransferase activities were concordantly affected (10).

Although heterodimerization may be necessary for native levels of methyltransferase, our results show that it is not sufficient. One of the mutants created in the present study displayed the unique property of retaining an ability to heterodimerize comparable with that of the wild-type D1 protein while failing to display any methyltransferase activity. This mutation, H682A,Y683A, defines for the first time a discrete site within the large subunit that is essential per se for catalysis of methyl group transfer. Another mutant, D620A,D622A with partial retention of D12 binding, is also catalytically inactive. The utility of such mutant proteins in dissecting the catalytic mechanism is considered below.
Catalytic domains are organized in a modular fashion within the heterodimeric vaccinia virus mRNA capping enzyme. The amino-terminal 60 kDa of the D1 subunit constitutes an autonomous functional unit containing both the RNA triphosphatase and RNA guanylyltransferase activities (7, 8). The RNA (guanine-7) methyltransferase domain is a distinct, non-overlapping, autonomous unit consisting of the carboxyl portion of the large subunit heterodimerized with the D12 protein (10). These two domains in the native enzyme are linked via a protease-sensitive hinge region of the large subunit (13). The significant finding of the present study is that the carboxyl portion of the large subunit has an intrinsic methyltransferase activity, which is dramatically stimulated by the small subunit. Thus, the active site of the methyltransferase must reside in the D1 polypeptide per se.

Previous studies of the activities associated with the D1 subunit were hampered by the low levels of soluble protein recov-

** FIG. 5. ** Methyltransferase activity of D1(540-844) expressed singly and together with D12. Methyltransferase reaction mixtures contained 19 fmol of cap-labeled poly(A) and the indicated amounts of glycerol gradient fractions of His-D1(540-844)/D12 or His-D12/D1(540-844) heterodimers (panel A) or His-D1(540-844) monomer (panel B).

** DISCUSSION **

Catalytic domains are organized in a modular fashion within the heterodimeric vaccinia virus mRNA capping enzyme. The amino-terminal 60 kDa of the D1 subunit constitutes an autonomous functional unit containing both the RNA triphosphatase and RNA guanylyltransferase activities (7, 8). The RNA (guanine-7) methyltransferase domain is a distinct, non-overlapping, autonomous unit consisting of the carboxyl portion of the large subunit heterodimerized with the D12 protein (10). These two domains in the native enzyme are linked via a protease-sensitive hinge region of the large subunit (13). The significant finding of the present study is that the carboxyl portion of the large subunit has an intrinsic methyltransferase activity, which is dramatically stimulated by the small subunit. Thus, the active site of the methyltransferase must reside in the D1 polypeptide per se.

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Fig. 7. Subunit interaction assessed by translation in vitro and immune precipitation. Synthetic mRNAs derived from wild-type or mutated His-D1(498–844) genes and the D12 gene were translated individually or cotranslated in a rabbit reticulocyte lysate. [35S]Methionine-labeled polypeptides were resolved by polyacrylamide gel electrophoresis. Precipitations with anti-D1(498–844) serum K643A plus D12 (lanes A4 and A12); E736A alone (lanes A7). Positions of the D1 and D12 subunit polypeptides are indicated at the right of each panel. The transcripts used to program the translation reactions were as follows: WT His-D1(498–844) alone (lanes A1, A10, B1, and B11); D12 alone (lanes A9, A15, C5, and C8); WT His-D1(896–844) plus D12 (lanes A2, A11, B2, and B12); K643A alone (lane A3), K643A plus D12 (lanes A4 and A12); E736A alone (lane A5); E736A plus D12 (lanes A6 and A13), D620A,D622A alone (lane A7), D620A,D622A plus D12 (lanes A8 and A14); L616A,V617A alone (lane A9), L616A,V617A plus D12 (lanes B4 and B13); G563A,P564A,G566A alone (lane B5); G566A,P564A,G566A plus D12 (lanes B6 and B14); D598A,G600A,G602A alone (lane B7), D598A,G600A,G602A plus D12 (lanes B8 and B15); G704A,G705A,V707A alone (lane B9); G704A,G705A,V707A plus D12 (lanes B10 and B16); T710A, T711A,M712A alone (lane C1); T710A,T711A,M712A plus D12 (lanes C2 and C6); H682A,Y683A alone (lane C3); H682A,Y683A plus D12 (lanes C4 and C7).

A Translation Products Immune Precipitates

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subunit when the subunits are coexpressed in vivo or in vitro, and the proteins can functionally interact when mixed in vitro. Reconstitution of methyltransferase by mixing the full-length D1 and D12 subunits had been demonstrated by Higman et al. (8). Our deletion analysis now shows that a 305-amino acid region of D1 from residues 540–844 is apparently sufficient for reconstitution of the methyltransferase domain. This same D1 protein segment has weak intrinsic methyltransferase and can heterodimerize with the small subunit in vitro.

Using different purification strategies and an alternative methyltransferase assay, Niles and co-workers (21) have also detected a low level of methyltransferase activity in preparations of D1(498–844) expressed in bacteria. In agreement with our results, they have found that methyltransferase activity is stimulated by association of D1(498–844) with the D12 protein. UV cross-linking of AdoMet to the methyltransferase domain by Higman and Niles (22) localized the sites of AdoMet binding to two fragments of the D1 subunit, one extending from amino acids 499–579 and a second from 801–844. Combining this finding with our demonstration of methyltransferase activity of the D1(540–844) protein and the complete loss of function upon further deletion to amino acid 579 leads to the conclusion that one site of AdoMet binding is situated between amino acids 540 and 579 and that this binding region is required for methyltransferase activity.

The application of alanine-scanning mutagenesis to the D1(498–844) protein has shed light on the role of specific protein sites in subunit association and enzyme activity. Because our choice of mutated residues was dictated by identity among three viral capping enzyme large subunits, we anticipated that many of our mutations would have functional consequences, and, indeed, seven of nine mutated proteins were defective for methyltransferase activity in the presence of the D12 subunit. In most cases, the lack of methyltransferase activity could be explained simply by the inability of the alanine-substituted D1 protein to heterodimerize with D12. Although the assay for subunit interaction, based on in vitro translation and coimmunoprecipitation, admittedly provides only a qualitative view of subunit affinity, the lack of subunit association in vitro has been predictive of lack of D12-dependent methyltransferase activity, in this study and in earlier analysis of truncated versions of the subunits. Unfortunately, we have not been able to use the in vitro translation approach to assess the effect of the alanine substitutions on the intrinsic methyltransferase activity of D1(498–844); this is because the activity was too low to be reliably detected in the reticulocyte lysate. Initial attempts to address the question by expressing the alanine-substituted His-D1(498–844) proteins in bacteria in the absence of the D12 subunit were obstructed by protein insolubility. Thus, it is not yet clear whether the amino acid residues implicated in subunit binding also play a role in catalysis.

The triple substitution D1 mutant D598A,G600A,G602A defines an essential region of the vaccinia methyltransferase domain that bears some resemblance to a motif UExD/E(IXG)XG (where U indicates an aliphatic amino acid and X is any amino acid) found in other AdoMet-requiring methyltransferases (23). Recently, this motif has been noted in proteins encoded by flaviviruses and by reovirus (24). The African swine fever virus large subunit contains a perfect match (VIDLGIGKG) to the consensus element, whereas the two poxvirus capping enzymes subunits lack the third alternating glycin residue. The motif is situated 20 amino acids away from the region shown to be one of the sites of AdoMet cross-linking to the vaccinia D1 protein (22). Our initial mutational analysis suggests that the motif is functionally relevant; creation of additional single substitution mutations in this region will be
useful in assessing the role of this motif in AdoMet utilization by the capping enzyme. Note that a variant of this sequence is conserved among DNA methyltransferases; that conserved element, referred to as "motif I," directly interacts with AdoMet in the cocystal of HhaI methyltransferase (25).

In the case of the H682A,Y683A mutation, we have created a protein specifically defective for methyltransferase activity but not for subunit interaction. The properties of this mutant protein seen in the in vitro translation experiments have been fully corroborated by coexpressing the His-D1(498-844) H682A,Y683A and D12 subunits in bacteria, i.e. we have purified the expressed protein as a 1:1 heterodimer and shown that the purified protein is defective for methyltransferase activity.

Therefore, we presume that the residues His-682-Tyr-683 constitute part of the methyltransferase active site. Future studies will focus on the interaction of this methyltransferase mutant with AdoMet and with capped RNA substrates. We anticipate that expanding the collection of alanine substitution mutations in the D1 protein and extending this approach to the D12 protein should provide a higher resolution map of the active site and the subunit interface.

REFERENCES
2. X. Mao, unpublished data.