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Mutational Analysis of mRNA Capping Enzyme Identifies Amino Acids Involved in GTP Binding, Enzyme-Guanylate Formation, and GMP Transfer to RNA

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Vaccinia virus mRNA capping enzyme is a multifunctional protein with RNA triphosphatase, RNA guanylyltransferase, RNA (guanine-7) methyltransferase, and transcription termination factor activities. The protein is a heterodimer of 95- and 33-kDa subunits encoded by the vaccinia virus D1 and D12 genes, respectively. The capping reaction entails transfer of GMP from GTP to the 5′-diphosphate end of mRNA via a covalent enzyme-(lysyl-GMP) intermediate. The active site is situated at Lys-260 of the D1 subunit within a sequence element, KxDG (motif I), that is conserved in the capping enzymes from yeasts and other DNA viruses and at the active sites of covalent adenylylation of RNA and DNA ligases. Four additional sequence motifs (II to V) are conserved in the same order and with similar spacing among the capping enzymes and several ATP-dependent ligases. The relevance of these common sequence elements to the RNA capping reaction was addressed by mutational analysis of the vaccinia virus D1 protein. Nine alanine substitution mutations were targeted to motifs II to V. Histidine-tagged versions of the mutated D1 polypeptide were coexpressed in bacteria with the D12 subunit, and the His-tagged heterodimers were purified by Ni affinity and phosphocellulose chromatography steps. Whereas each of the mutated enzymes retained triphosphatase, methyltransferase, and termination factor activities, six of nine mutant enzymes were defective in some aspect of transguanylylation. Individual mutations in motifs III, IV, and V had distinctive effects on the affinity of enzyme for GTP, the rate of covalent catalysis (EpG formation), or the transfer of GMP from enzyme to RNA. These results are concordant with mutational studies of yeast RNA capping enzyme and suggest a conserved structural basis for covalent nucleotidyl transfer.

Capping of eukaryotic mRNAs occurs by a series of three enzymatic reactions in which the 5′ triphosphate-terminated primary transcript is converted to a diphosphate-terminated RNA by RNA triphosphatase, capped with GMP by RNA guanylyltransferase, and then methylated by RNA (guanine-7-) methyltransferase. All three steps in cap formation are catalyzed by the vaccinia virus capping enzyme, a heterodimeric protein composed of 95- and 33-kDa subunits encoded by the vaccinia virus D1 and D12 genes, respectively (6, 19, 21, 22, 28, 37, 42). Catalytic domains are organized in a modular fashion within the vaccinia virus enzyme. The amino-terminal 60 kDa of the D1 subunit constitutes an autonomous functional unit containing both the triphosphatase and guanylyltransferase activities (10, 27, 33). The methyltransferase domain is a distinct, nonoverlapping, autonomous unit consisting of the carboxyl portion of the large subunit heterodimerized with the D12 protein (1, 11, 17).

The guanylyltransferase component of the enzyme catalyzes two sequential nucleotidyl transfer reactions involving a covalent enzyme-guanylate intermediate (31). In the first partial reaction, nucleophilic attack on the α-phosphate of GTP by enzyme results in liberation of PP_i and formation of a covalent adduct (EpG) in which GMP is linked via a phosphoamide bond to the ε-amino group of a lysine residue (Lys-260) of the D1 subunit (2, 20). The nucleotide is then transferred to the 5′ end of the RNA acceptor to form an inverted (5′)-(5′) triphosphate bridge structure. An identical covalent mechanism applies to all cellular guanylyltransferases and to several other viral capping enzyme systems (29, 36).

The RNA capping reaction is mechanistically similar to enzyme-mediated DNA and RNA ligation reactions (31, 37). The polynucleotide ligases attack the α-phosphate of ATP or NAD to form a covalent intermediate (EpA) in which AMP is linked to the ε-amino group of a lysine (14). The AMP is then transferred to the 5′ monophosphate end of a polynucleotide to form an inverted (5′)-(5′) diphosphate bridge structure, AppN, which resembles the unmethylated RNA cap.

A potential common structural basis for covalent nucleotidyl transfer was illuminated by peptide mapping and mutational analyses of the active sites of several polynucleotide ligases (2, 9, 13, 35, 39, 40, 43, 45) and RNA guanylyltransferases (2, 5, 20, 25, 32). The active-site Lys of the vaccinia virus capping enzyme is situated within a motif, KxDG, that is conserved among the guanylyltransferases encoded by *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, Shope fibroma virus, and African swine fever virus (Fig. 1). The KxDG motif is also found at the active sites of mammalian DNA ligases I and II, vaccinia virus DNA ligase, T4 RNA ligase, and yeast tRNA ligase. The Lys and Gly residues in the KxDG motif are strictly essential for EpG formation by the capping enzymes of vaccinia virus and *Saccharomyces cerevisiae* (2, 5, 32) and for EpA formation by human DNA ligase I (13). Even subtle substitutions at these positions abolish covalent catalysis. That the active sites of capping enzymes and ligases are so similar suggested that other structural features might be conserved. Indeed, we have detected local sequence conservation at four additional motifs (designated II to V) downstream of the KxDG element (hereafter referred to as motif I) (32). What is most remarkable about these sequence motifs is that they are arranged in the same order, and with nearly identical spacing, in the capping enzyme and ATP-dependent ligase family mem-

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	I		II		III		IV		V
			*		** *		**		* * *
VAC CE	KTDG	-23-	RYP	-8-	VVVFGEAV	-69-	EGVIL	-10-	DFKIKKENTID
SFV CE	KTDG	-23-	RYD	-8-	VTLYGEAV	-68-	EGVVL	-9-	DYKIKLDNTTD
ASF CE	KADG		-32-		TILDGEFM	-78-	DGIIL	-13-	KWKPTWDNTLD
Sc CE	KTDG	-33-	RFP	-17-	TLLDGELV	-90-	DGLIF	-17-	KWKPEQENTVD
Sp CE	KSDG	-34-	FYP	-13-	TLLDGELV	-90-	DGLIF	-16-	KWKPKEMNTID
Dam DNA	KYDG	-24-	QYP	-14-	FIVEGEII	-96-	EGVMV	-17-	WIKFKRDYQSE
Sc DNA	KYDG	-25-	RYP	-16-	LILDCEAV	-96-	EGLMV	-18-	WLKLKKDYLEG
Sp DNA	KYDG	-25-	RYP	-16-	FILDCEAV	-96-	EGLMV	-18-	WLKVKKDYLSG
Hu1 DNA	KYDG	-25-	KYP	-16-	FILDTEAV	-96-	EGLMV	-17-	WLKLKKDYLDG
Hu3 DNA	KYDG		-43-		MILDSEVL	-92-	EGLVI	-13-	WLKVKKDYLNE
Hu4 DNA	KLDG		-49-		CILDGEMM	-93-	EGIMV	-14-	WLKIKPEYVSG
VAC DNA	KYDG		-43-		IVLDSEIV	-91-	EGLVL	-13-	WLKIKRDYLNE
SFV DNA	KYDG		-43-		FILDAELV	-92-	EGLML	-13-	WLKIKKDHLKT
FPV DNA	KYDG		-43-		MILDGEII	-92-	EGFVL	-13-	WLKIKKDYLDG
ASF DNA	KRNG		-43-		VYLDGELY	-85-	EGAIV	-20-	KLKPLLDAEFI
T4 DNA	KADG		-49-		VLIDGELV	-124-	EGIIL	-16-	KFKEVIDVDLK
T4 RNA	KEDG		-4-		TYLDGDEI	-112-	EGYVA	-6-	HFKIKSDWYVS

FIG. 1. Regions of conservation between capping enzymes and polynucleotide ligases. Five collinear sequence elements, designated motifs I to V, are conserved in capping enzymes and polynucleotide ligases as shown. The amino acid sequences are aligned for capping enzymes (CE) encoded by vaccinia virus (VAC), Shope fibroma virus (SFV), African swine fever virus (ASF), *S. cerevisiae* (Sc), and *Schizosaccharomyces pombe* (Sp) (see references 21, 24, 26, 32, and 41 for complete capping enzyme sequences). Aligned in the lower group are DNA ligases (DNA) from *Desulfurolobus ambivalens* (Dam), *S. cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), human ligase I (Hu1), human ligase III (Hu3), human ligase IV (Hu4), vaccinia virus (VAC), Shope fibroma virus (SFV), fowlpox virus (FPV), African swine fever virus (ASF), and bacteriophages T4 and T3. (The complete sequences can be found in references 12, 23, 38, and 44.) Also included in the polynucleotide ligase family alignment is T4 RNA ligase. The number of intervening amino acid residues is indicated (*-n-*). Residues in the vaccinia virus capping enzyme that were targeted for mutational analysis are indicated by asterisks above the aligned sequence.

bers (Fig. 1). We have proposed that these motifs define a phylogenetically related superfamily of covalent nucleotidyl transferases (32, 36).

Is sequence conservation between capping enzymes and ligases relevant to their common catalytic mechanism, and, if so, how? To address these questions, Ala substitution mutations were targeted to individual residues within motifs II to V of the vaccinia virus capping enzyme D1 subunit. The multifunctional nature of the vaccinia virus protein makes it especially well suited to biochemical assessment of structure-function relationships, for the following reasons: (i) the D1 and D12 subunits can be coexpressed in bacteria (6, 28) and purified rapidly by affinity chromatography (15–17); (ii) the recombinant heterodimeric enzyme is functionally indistinguishable from the enzyme purified from vaccinia virions; (iii) testing the mutated proteins for each of the known activities of the capping enzyme makes it possible to distinguish mutational effects on global protein structure (which might be expected to affect multiple functions) from those that are specific for only one of the catalytic activities (15); and (iv) mutational effects on the guanylyltransferase partial reactions can, in principle, be discriminated and quantitated.

MATERIALS AND METHODS

T7-based plasmids for expression of vaccinia virus capping enzyme. Plasmid pET-His_{10}-D1 contains the entire D1 coding sequence fused in frame with a 63-bp 5′ leader sequence that encodes 10 consecutive histidine residues. The *Eco*RI site in the parent pET vector was deleted prior to construction of pET-His_{10}-D1; thus, this plasmid contains only a single *Eco*RI site within the D1 gene. Alanine substitution mutations in the D1 gene were programmed by synthetic oligonucleotides via the two-stage PCR-based overlap extension strategy, as described previously (2). A 1-kbp *Stu*I-*Eco*RI restriction fragment of the PCR-amplified DNAs that included the mutated region was exchanged for the corresponding segment of wild-type pET-His_{10}-D1, thereby generating a series of pET-His-D1-Ala expression plasmids. The presence of the desired mutation was confirmed in each case by sequencing the entire 1-kbp insert; the occurrence of PCR-generated mutations outside the targeted region was thereby excluded. A series of coexpression plasmids—pET-D12/His_{10}-D1—was engineered by excision of the wild-type and Ala-substituted His_{10}-D1 cassettes with *Bgl*II and *Hin*dIII and insertion into pET-D12 that had been cut with *Bam*HI and *Hin*dIII.

Capping enzyme expression and purification. *Escherichia coli* BL21(DE3) bearing wild-type or mutated pET-D12/His_{10}-D1 plasmids was inoculated into Luria-Bertani medium containing 0.1 mg of ampicillin per ml and grown at 37°C until the A_{600} reached approximately 0.6. Cultures (400 ml) were then placed on ice for 30 min, adjusted to 2% ethanol, and subsequently incubated at 18°C for 48 h with continuous shaking. Cells were harvested by centrifugation, and the pellets were stored at −80°C. All subsequent procedures were performed at 4°C. The cells were lysed by treatment of thawed, resuspended cells with lysozyme (0.2 mg/ml)–Triton X-100 (0.1%) in 20 ml of lysis buffer (50 mM Tris HCl [pH 7.5], 0.15 M NaCl, 10% sucrose). Insoluble material was removed by centrifugation at 18,000 rpm in a Sorvall SS-34 rotor for 40 min. The supernatants were mixed with 1 ml of Ni-nitrilotriacetic acid-agarose resin (Qiagen) for 1 h. The slurries were 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 phenylmethylsulfonyl fluoride, 10% glycerol) containing 5, 50, and 200 mM imidazole. The polypeptide composition of the column fractions was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions enriched for the expressed capping-enzyme subunits (which were eluted at 200 mM imidazole) were pooled and then dialyzed against buffer A (50 mM Tris HCl [pH 8.0], 2 mM EDTA, 2 mM dithiothreitol, 0.1% Triton X-100, 10% glycerol). Each dialysate was applied to a 2-ml column of phosphocellulose that had been equilibrated with buffer A. The columns were eluted stepwise with buffer A containing 0.05, 0.1, 0.5, and 1 M NaCl. The D1-D12 heterodimers were recovered in the 0.5 M NaCl fraction. The protein concentration of the phosphocellulose capping-enzyme fractions was determined by using the Bio-Rad dye reagent with bovine serum albumin as a standard.

Assay of enzyme-GMP complex formation. Reaction mixtures (20 μl) containing 50 mM Tris HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, [α-^{32}P]GTP as indicated, and capping enzyme were incubated for 5 min at 25°C. Reactions were halted by the addition of SDS to 1%, and the samples were then analyzed by electrophoresis through a 10% polyacrylamide gel containing 0.1% SDS. Labeled polypeptides were visualized by autoradiography of the dried gel. The extent of covalent-complex formation was quantitated by liquid scintillation counting of an excised gel slice or by scanning the dried gel with a FUJIX BAS1000 phosphorimager.

Methyltransferase assay. Reaction mixtures (10 μl) contained 50 mM Tris HCl (pH 7.5), 5 mM dithiothreitol, 50 μM *S*-adenosylmethionine, 29 fmol of ^{32}P-cap-labeled poly(A), and enzyme. After incubation at 37°C for 5 min, the samples were adjusted to 50 mM sodium acetate (pH 5.5) and digested with 5 μg of nuclease P1 for 60 min at 37°C. Cap dinucleotide (GpppA) and methylated cap dinucleotide (m7GpppA) were resolved by thin-layer chromatography on polyethyleneimine cellulose plates that were developed with 0.35 M $(NH_4)_2SO_4$. The extent of cap methylation was quantitated by scanning the chromatogram with a phosphorimager.

Triphosphatase assay. Activity was determined by the release of $^{32}P_i$ from [γ-^{32}P]ATP as previously described (37). Reaction mixtures (50 μl) containing 60 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, and enzyme were incubated for 30 min at 37°C.

RESULTS

Mutagenesis strategy. Five sequence motifs within the guanylyltransferase domain of the vaccinia virus D1 protein are arrayed in the same order and with similar spacing in the capping enzymes of other DNA viruses and in the guanylyltransferases from budding and fission yeasts (Fig. 1). The same motifs are found in ATP-dependent polynucleotide ligases from multiple sources (Fig. 1). Our initial mutational analysis established the essentiality of residues Lys-260 and Gly-263 in motif I of the vaccinia virus D1 protein; substitutions at these positions abolished enzyme-GMP complex formation in vitro (1). In the present study, we introduced mutations at nine conserved positions in motifs II to V. Residues mutated included Tyr-288 in motif II; Gly-302, Glu-303, and Val-305 in motif III; Glu-375 and Gly-376 in motif IV; and Lys-392, Thr-398, and Asp-400 in motif V (these residues are denoted by asterisks in Fig. 1). In each case, the wild-type amino acid was replaced by alanine. Because alanine substitution eliminates the side chain beyond the β-carbon, usually without perturbing global protein structure, this mutational approach provides an indication of the essentiality of the side chain for enzyme activity.

His-tagged versions of the wild-type and Ala-substituted D1 proteins were expressed in bacteria together with the D12 capping enzyme subunit. The coexpressed D1 and D12 proteins normally form a stable heterodimer when coexpressed in bacteria (28). The Ala-substituted His-tagged enzymes were purified by sequential Ni affinity and phosphocellulose chromatography steps. SDS-PAGE analysis of the phosphocellulose preparations revealed that both capping enzyme subunits were present in seemingly equimolar amounts in every case (Fig. 2A). Thus, none of the mutations affected subunit heterodimerization. The D1 and D12 subunits were the predominant polypeptides in each enzyme preparation (Fig. 2A).

A preliminary test for mutational effects on guanylyltransferase activity was performed by incubating equivalent amounts of input protein from each phosphocellulose preparation with 0.17 μM [α-^{32}P]GTP and magnesium; these are conditions under which the wild-type enzyme readily formed the covalent enzyme-guanylate intermediate. EpG formation was detected by label transfer to the 97-kDa His-D1 polypeptide (Fig. 2B). The extent of EpG formation was linear with respect to input protein. Specific activities of the mutated enzymes relative to the wild type were determined via titration experiments performed within the linear range (Table 1). Mutations E375A and G376A in motif IV and mutations K399A, T398A, and D400A in motif V adversely affected EpG formation. In contrast, the Ala substitutions in motifs II and III were relatively well tolerated.

Mutational effects on affinity for GTP. The guanylyltransferase reaction can be viewed as the sum of multiple discrete steps. At a minimum, these steps include (i) noncovalent binding of GTP to enzyme to form an E · GTP binary complex; (ii) covalent reaction chemistry, whose immediate product is EpG · PP_i; (iii) dissociation of PP_i; (iv) binding of the diphosphate-terminated RNA cap acceptor to guanylylated enzyme to form EpG · ppRNA; (v) nucleotidyl transfer from enzyme to RNA to yield E · GpppRNA; and (vi) dissociation of the capped RNA product. Each of these steps is freely reversible (18, 31).

Our initial experiments suggested that five of the Ala mutations affected EpG formation. Hence, these mutations must elicit a defect in at least one of the first three steps in this scheme. To determine whether any of the mutant capping enzymes might display altered affinity for GTP, we tested the effect of increasing GTP concentration on enzyme-guanylate formation (Fig. 3). An equivalent amount of input protein was used in each titration series. The yield of EpG by wild-type capping enzyme increased with GTP concentration up to 0.5 μM and leveled off thereafter. The extent of EpG formation at 0.2 μM GTP was about 60% of that at 10 μM GTP (Fig. 3). A profound effect on GTP-binding affinity was elicited by the E375A, K392A, and D400A mutations (Fig. 3B); the titration profiles of these proteins were shifted to the right by at least an order of magnitude. EpG formation by these three proteins was minimal at the low nucleotide concentrations (0.17 μM) used in our screening assays; however, in the case of K392A and E375A, the extent of EpG formation at 10 μM approached or exceeded that of the wild type.

The remaining mutations had either no effect or only a slight

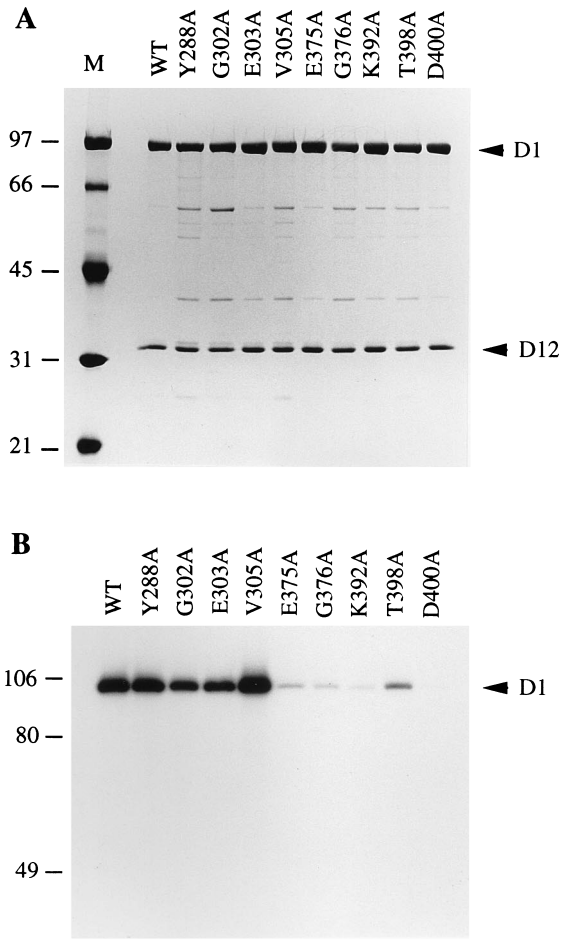

FIG. 2. Purification of wild-type and mutated capping enzymes. (A) The polypeptide composition of the phosphocellulose capping-enzyme fractions was determined by SDS-PAGE; 3 μg of protein was applied to each lane of a 10% polyacrylamide gel. The identity of the recombinant protein is indicated above the lanes. Polypeptides were visualized by staining with Coomassie blue. The polypeptides corresponding to the D1 and D12 capping-enzyme subunits are indicated on the right by arrowheads. The sizes (kilodaltons) of coelectrophoresed marker polypeptides (lane M) are indicated on the left. (B) Enzyme-GMP complex formation. Reaction mixtures (20 μl) containing 50 mM Tris HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.17 μM [α-^{32}P]GTP (3,000 Ci/mmol), and 140 ng of protein (phosphocellulose fraction) were incubated for 5 min at 25°C, denatured, and analyzed by SDS-PAGE. An autoradiograph of the dried gel is shown. The identity of the recombinant enzyme is indicated above each lane. The position of the GMP-labeled D1 subunit is indicated on the right by an arrowhead. The positions and sizes (kilodaltons) of coelectrophoresed prestained marker polypeptides are shown on the left.

TABLE 1. Specific activities of wild-type and mutated enzymes

Motif	Mutant	Sp act (% of wild type)[a]:			
		EpG formation		Methyl-transferase	ATPase
		0.17 μM GTP	10 μM GTP		
II	Y288A	116	ND[b]	82	85
III	G302A	44	50	146	81
	E303A	98	156	201	149
	V305A	216	208	222	123
IV	E375A	11	135	283	169
	G376A	7	21	104	50
V	K392A	5	91	409	115
	T398A	16	11	89	51
	D400A	1	17	234	92

[a] Wild-type and mutant capping enzymes were assayed for enzyme-guanylate formation at 0.17 μM or 10 μM [α-^{32}P]GTP; the extent of label transfer to the D1 subunit was determined at three levels (1×, 2×, and 3×) of each enzyme fraction within the linear response range. Methyltransferase reaction mixtures contained 29 fmol of cap-labeled poly(A); m7GpppA formation was determined at five levels (1×, 2×, 4×, 8×, and 16×) of each enzyme fraction. The wild-type protein methylated 21 nmol of RNA per mg of protein. Triphosphatase assay mixtures contained 1 mM [γ-^{32}P]ATP and three levels of (1×, 2×, and 4×) of each enzyme. The wild-type enzyme released 24 μmol of P_i per mg of protein. Specific activities of the mutant enzymes are expressed as percentages relative to those of the wild-type enzyme.

[b] ND, not determined.

effect on the affinity for GTP (as judged by the shape of the titration curve), but the mutations differentially affected the yield of EpG. For example, the V305A enzyme displayed a GTP dependence similar to that of the wild type, but the absolute amount of EpG formed in this experiment was more than twice that of the wild type (Fig. 3A). The GTP titration profiles of the G302A and T398A proteins also paralleled those of the wild type, but the extent of covalent adduct was significantly lower. The E303A protein displayed a mild reduction in affinity for GTP, but the yield at saturation was higher than that of the wild type (Fig. 3A). The G376A mutation also shifted the titration curve slightly, but in this case the yield at saturation was far less than that of the wild type (Fig. 3B).

In light of these experiments, we repeated the determination of the specific activities of the mutant proteins at 10 μM [α-^{32}P]GTP; the activities were normalized to that of the wild-type enzyme and are shown in Table 1. Simple comparison of the relative specific activities at 10 and 0.17 μM GTP readily identified the E375A, K398A, and D400A proteins as having reduced affinity for GTP; the magnitude of the defect was roughly indicated by the ratio of the high-GTP to low-GTP activity (12- to 18-fold for this class of mutants). The G376A mutation had a modest effect (threefold) on GTP binding. All four residues implicated in the nucleotide-binding step are situated within motifs IV and V.

Mutational effects on the rate of EpG formation. We performed a kinetic analysis of EpG formation by wild-type and mutant capping enzymes in the presence of 10 μM [α-^{32}P]GTP. Each time course reaction mixture included an equal amount of input protein. The wild-type reaction was virtually complete within 10 s at 25°C (Fig. 4). Clearly, the approach to equilibrium was too rapid to determine a first-order rate constant without using a rapid-mixing/quenching apparatus. Nonetheless, we were able to discern two cases—E375A and K392A—in which Ala substitution clearly and profoundly affected the rate of covalent catalysis without altering the yield of the covalent adduct

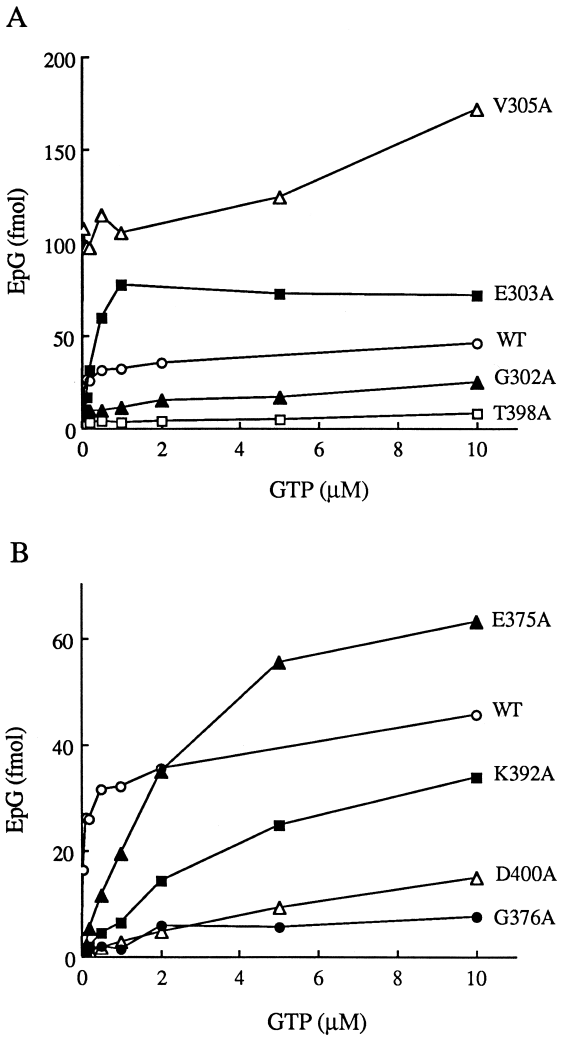

FIG. 3. EpG formation as a function of GTP concentration. Reaction mixtures contained 50 mM Tris HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, 34 ng of capping enzyme (phosphocellulose fraction), and [α-^{32}P]GTP as indicated. Incubation was carried out for 5 min at 25°C. The extent of enzyme-guanylate formation is plotted as a function of GTP concentration for the wild-type (WT) and each mutant enzyme, as indicated.

(Fig. 4B). EpG formation by the E375A and K392A proteins was essentially linear over the 160-s time course, and simple extrapolation to the wild type indicates a >10-fold effect on the reaction rate. (The true magnitude of the rate effect is probably much higher but cannot be assessed without finer analysis of the wild-type rate.) The G376A mutation caused a more modest decrease in rate; EpG formation was linear for 20 s and plateaued at 40 to 60 s (Fig. 4B). The level of EpG was much lower than that of the wild type, consistent with the specific activity data in Table 1. An even milder rate effect was seen for E303A; the level of EpG plateaued at 20 to 30 s and was higher than that of the wild type (Fig. 4A); this is also in agreement with the specific activity data of Table 1. The remaining mutant proteins showed a kinetic profile similar to that of the wild type but varied with respect to the yield of EpG at equilibrium. Note that the sensitivity of the kinetic measurements is such that we probably could not detect rate effects of a fewfold that would be manifest only at time points shorter than 10 s.

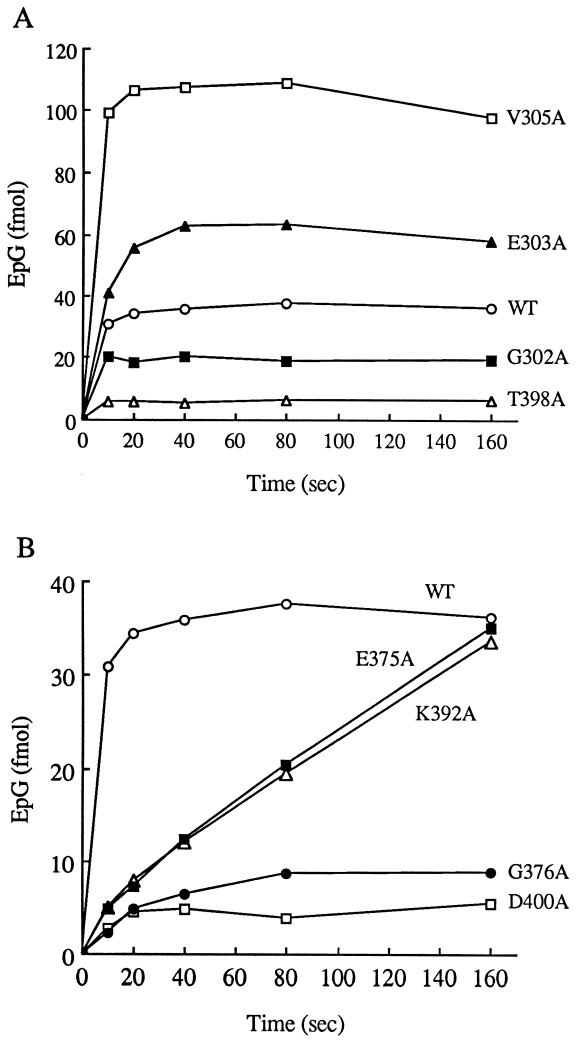

FIG. 4. Time course of EpG formation by wild-type and mutated capping enzymes. A reaction mixture (120 μl) containing 50 mM Tris HCl (pH 8.0), 5 mM dithiothreitol, 5 mM $MgCl_2$, 10 μM [α-^{32}P]GTP, and 204 ng of enzyme was incubated at 25°C. Aliquots (20 μl) were withdrawn at 10, 20, 40, 80, and 160 s; the samples were added immediately to 20 μl of 2× SDS gel sample buffer to terminate the reaction. The extent of enzyme-guanylate formation is plotted as a function of incubation time for the wild-type (WT) and each mutant enzyme, as indicated.

Targeted mutations selectively affect the guanylyltransferase component of the capping enzyme. Rigorous interpretation of mutational effects on the guanylyltransferase component demands that we distinguish global effects on protein structure and function from those specific to individual domains. We showed previously that the triphosphatase domain of the capping enzyme colocalizes with the guanylyltransferase to the N-terminal 60 kDa of the D1 subunit, whereas the methyltransferase domain is a distinct unit composed of the C-terminal portion of the D1 subunit complexed with the full-length D12 subunit (1, 2, 17, 33). We would expect that mutations in motifs II to V of the D1 subunit (within the N-terminal segment) should not inactivate the methyltransferase unless they disrupt the structure of the C-terminal segment. Methyltransferase activity was assayed as the *S*-adenosylmethionine-dependent conversion of cap-labeled poly(A) to methylated cap-labeled poly(A). The extent of cap methylation was linear with respect to input protein for all preparations. None of the mutations caused significant reduction in specific activity (Table 1), thereby ruling out gross structural defects.

The triphosphatase activity of the capping enzyme was assayed by the release of $^{32}P_i$ from 1 mM [γ-^{32}P]ATP. ATP hydrolysis increased linearly with time during a 30-min incubation. The reaction rate was linear with respect to input protein for the wild-type capping enzyme and for each of the Ala mutants. The specific activities of the mutant capping enzymes (per microgram of protein) were all comparable to that of the wild-type protein (Table 1). Thus, none of the Ala substitutions significantly affected the triphosphatase component of the capping enzyme. The apparent small differences in specific ATPase activity among the Ala mutants (±50% relative to the wild type) may be caused by a combination of (i) variable activity loss during the purification procedure and (ii) slight variations in the abundance of minor contaminating polypeptides in the phosphocellulose fraction (which can affect the calculated specific activity merely by changing the percentage of total protein that is contributed by the D1 subunit).

We suggest that the ATPase activity is the most reliable measure of the level of catalytically active D1 subunit in the capping enzyme preparations. Thus, the magnitude and selectivity of the effects of Ala substitutions on the EpG formation reaction can be inferred from the ratio of EpG formation and ATPase specific activities in Table 1. By this measure, we conclude that the Y288A, G302A, E303A, and V305A mutations did not significantly inhibit EpG formation (we arbitrarily defined the threshold of significance as a twofold effect on the ratio of EpG to ATPase). The E375A and K392A mutations did not significantly affect EpG formation at saturating GTP concentrations. A 60 to 80% reduction in EpG formation was observed for the G376A, T398A, and D400A mutations.

Mutational effects on RNA capping. Formation of the enzyme-GMP intermediate is only the first of two nucleotidyl transfer steps in the RNA capping reaction. Conceivably, mutations that have little impact on EpG formation may yet be defective in transfer of the covalently bound GMP from enzyme to RNA. Similarly, mutant proteins that form EpG less efficiently than the wild type does (e.g., those mutated in motifs IV and V) may or may not be affected in the RNA transfer step. To address this issue, we tested each preparation for RNA capping activity. This was assayed by the incorporation of [^{32}P]GMP from [α-^{32}P]GTP into a poly(A) cap acceptor. To focus specifically on the second nucleotidyl transfer step, we included in each reaction mixture an equivalent amount of capping enzyme as EpG-forming units and performed the reactions at 25 μM [α-^{32}P]GTP. The incorporation of labeled GMP into acid-insoluble material was dependent on the addition of poly(A). The RNA cap acceptor was present in excess over input enzyme; a molar excess of GMP was transferred to RNA by the wild-type enzyme during a 30-min reaction at 37°C (Fig. 5B). The poly(A) cap acceptor used in these experiments was triphosphate terminated. It is well established that the triphosphatase activity of the capping enzyme is capable of converting all the triphosphate ends to diphosphate ends at a rate that vastly exceeds that of RNA capping (37, 42); hence, the guanylyltransferase reaction is rate limiting under these conditions. Because none of the Ala mutants was defective in triphosphatase activity with ATP, we considered it unlikely that any mutational effects on capping would be caused by defects in γ-phosphate hydrolysis of the input poly(A). However, to address the issue directly, we performed control reactions containing [γ-^{32}P]triphosphate-terminated poly(A) and enzyme at the same concentrations used in the RNA capping assays. We found that each of the mutant enzymes catalyzed near-quan-

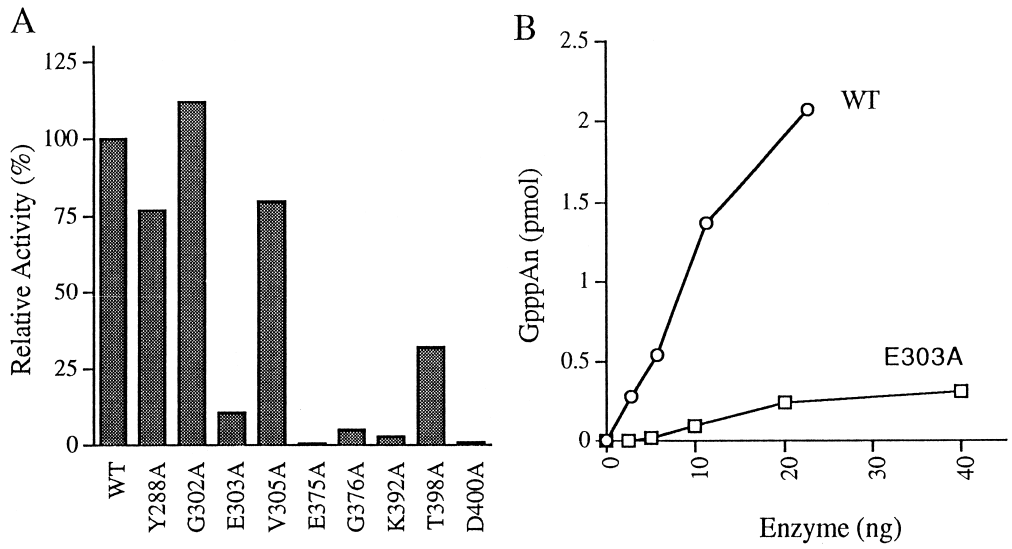

FIG. 5. RNA capping activity of mutated capping enzymes. (A) Reaction mixtures (20 μl) contained 50 mM Tris HCl (pH 7.5), 1.25 mM $MgCl_2$, 5 mM dithiothreitol, 25 μM [α-^{32}P]GTP, 11 pmol (of ends) of triphosphate-terminated poly(A), and either wild-type (WT) or mutant enzymes in such quantity as to constitute an equivalent amount (32 fmol) of EpG-forming activity. After incubation for 30 min at 37°C, reactions were halted by the addition of 5% trichloroacetic acid. Acid-insoluble material was collected by filtration, and incorporation of labeled nucleotide was quantitated by liquid scintillation counting. The extent of capping by each mutant protein is expressed relative to that of the wild-type enzyme (results shown are the average of two experiments). (B) Incorporation of GMP into poly(A) is plotted as a function of protein concentration for the wild-type (WT) and E303A mutant enzymes.

titative release of $^{32}P_i$ from the RNA substrate within the first 2 min of incubation at 37°C (results not shown).

The effects of the Ala substitutions on the guanylyltransferase reaction are shown in Fig. 5A. The Y288A and V305A proteins were 77 to 80% as active as the wild-type enzyme in the complete RNA capping reaction, and the G302A enzyme was at least as active as the wild type. Thus, these three mutations, which had little or no adverse affect on EpG formation, also had no significant effect on the second catalytic step. In contrast, the E303A mutation, which did not affect EpG formation, inhibited RNA capping by an order of magnitude (Fig. 5A). This was confirmed by a protein titration experiment comparing RNA capping by wild-type and E303A proteins (Fig. 5B). Thus, the E303A mutation specifically affected the transfer of GMP to RNA. The T398A protein displayed an intermediate level of RNA capping when normalized for EpG formation. This mutation apparently affects both nucleotidyl transfer steps; the magnitude of the second-step defect (32% of wild type) is similar to that of EpG formation (one-fifth of wild type after normalization to ATPase). The E375A, G376A, K392A, and D400A proteins were, respectively, 0.6%, 5%, 3%, and 1% as active as the wild-type enzyme in cap transfer to RNA. Although each of these proteins displayed some defect in EpG formation (either in affinity for GTP or in the rate of EpG formation), the mutations appeared to have greater quantitative impact on GMP transfer to RNA.

Y288A and G302A capping enzymes are thermolabile in vitro. The data in Table 1 and Fig. 5 indicated that three of the nine Ala mutations—Y288A, G302A, and V305A—had no major effect on guanylyltransferase activity in vitro. Whereas analogous mutations in motifs II and III of the *S. cerevisiae* guanylyltransferase were reported to be active in vivo, two of these (an RFP → AAA triple mutant in motif II and a single Gly → Ala substitution in motif III) conferred a temperature-sensitive growth phenotype (32). We therefore examined the stability of the vaccinia virus Y288A and G302A mutant enzymes in vitro. Wild-type and mutant enzyme preparations were heated for 5 min at either 30, 35, 40, 45, or 50°C, followed by quenching on ice. The samples were then assayed for EpG formation at 25°C. The wild-type guanylyltransferase activity was stable up to 40°C, was partially inactivated at 45°C, and was completely inactivated at 50°C (Fig. 6). The Y288A and G302A enzymes were both thermolabile in vitro, as reflected by a shift to the left of the heat inactivation curve (Fig. 6). It is not simply the case that any Ala substitution conferred thermolability, because EpG formation by the E303A mutant enzyme displayed a heat inactivation profile virtually identical to that of the wild-type enzyme (Fig. 6). Thus, the studies of both yeast and vaccinia virus capping enzyme suggest that some conserved residues, while not strictly essential for catalysis, may nonetheless contribute to stability of the enzyme in vivo and in vitro.

Transcription termination factor activity of mutant capping enzymes. In addition to its role in RNA 5′-end modification, the capping enzyme acts as a transcription termination factor during the synthesis of vaccinia virus early mRNAs (30, 34). The structural requirements for the viral termination factor (VTF) activity of the capping enzyme are distinct from those for nucleotidyl transfer and methyl transfer; i.e., mutations that completely abrogate guanylyltransferase activity (e.g., a substitution at the Lys-260 active site) or methyltransferase activity have no impact on VTF activity in vitro (15). Moreover, whereas the three capping domains have been assigned to individual subunits or portions thereof, both full-length enzyme subunits are required for VTF (15). How the N terminus of the D1 subunit contributes to transcription termination is unclear. Hence, we asked whether any of the Ala mutants described above might inhibit VTF activity.

VTF-dependent termination by vaccinia virus RNA polymerase was assayed during a single round of transcription programmed by a bead-linked template containing an early promoter fused to a 20-nucleotide G-less cassette. (This in vitro system has been described in detail in references 4 and 7.) Elongation by vaccinia virus RNA polymerase is arrested at

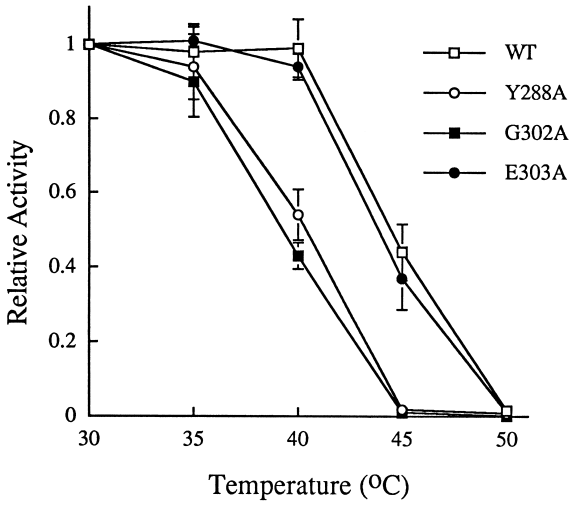

FIG. 6. Mutational effects on thermal stability. Aliquots (10 μl) of the wild-type (WT), Y288A, G302A, and E303A capping enzymes were heated for 5 min at either 30, 35, 40, 45, or 50°C and then placed on ice. The residual activity in enzyme-guanylate formation was assayed in reaction mixtures containing 50 mM Tris HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.17 μM [α-^{32}P]GTP, and 1 μl of the preincubated enzyme. After incubation for 5 min at 25°C, the samples were denatured and then resolved by SDS-PAGE. EpG formation was quantitated with a phosphorimager. Residual activity of each enzyme (expressed relative to that of the sample that had been preincubated at 30°C) is plotted as a function of temperature during the heating step. The data shown are the average of four separate heat inactivation experiments. Bars represent the mean standard error.

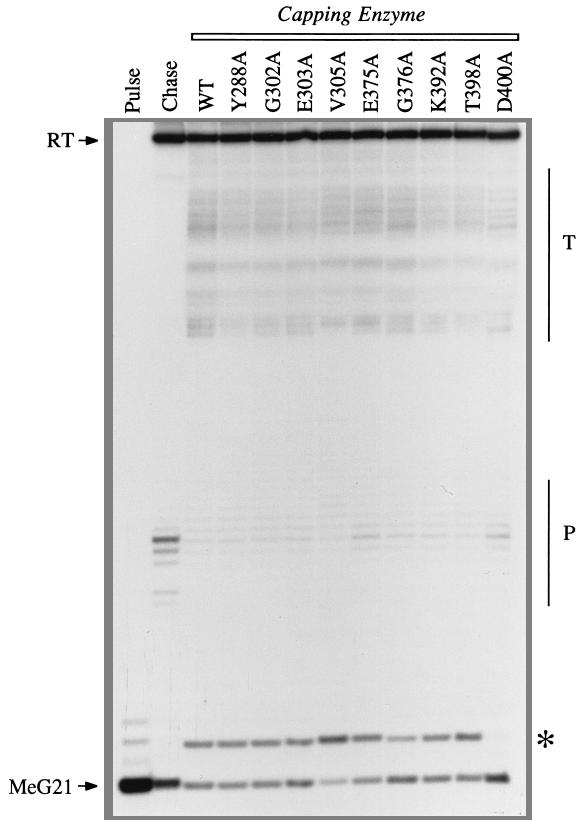

FIG. 7. Transcription termination by mutant capping enzymes. Ternary transcription complexes were formed as described previously (4, 7) in standard reaction mixtures containing 20 mM Tris HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM UTP, 1 μM [α-^{32}P]CTP (1,000 Ci/mmol), 0.1 mM 3′-*O*-methyl GTP, vaccinia virus RNA polymerase (holoenzyme fraction containing the early transcription initiation factor), and bead-linked G21(ter) DNA template. Reaction mixtures were incubated at 30°C for 10 min and concentrated by microcentrifugation for 20 s. The beads were held in place with an external magnet while the supernatant was removed, and the beads were washed three times with 0.1 ml of 20 mM Tris HCl (pH 8.0)–2 mM dithiothreitol. The beads were resuspended in a small volume of the wash buffer, and aliquots were distributed into individual reaction tubes. Elongation reactions (chase phase) were performed with 20-μl mixtures containing 20 mM Tris HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM each ATP, GTP, CTP, and UTP. Where indicated, 25 ng of capping enzyme was added to the chase reaction mixtures immediately prior to the NTPs. After incubation at 30°C for 5 min, reactions were stopped by addition of buffer containing SDS and urea. Samples were extracted with phenol-chloroform, and labeled RNA was recovered by ethanol precipitation. Transcription products were analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea, and radiolabeled transcripts were visualized by autoradiography. The positions of pulse-labeled 3′-*O*-methyl G21 RNA (MeG21) and the chased runoff transcripts (RT) are indicated by arrows on the left of the autoradiogram. Other RNA populations are denoted as follows: T, transcripts terminated at heterogeneous sites downstream of the termination signal (these terminated RNAs are capping enzyme dependent); P, paused transcripts, i.e., RNAs engaged within transcription complexes paused within the T-rich termination signal (7); *, released 21-mer chains that were guanylylated by the added capping enzyme.

template position G21 when 3′-*O*-methyl GTP is included in lieu of GTP in the reaction mixtures. Situated downstream of the G-less cassette is a transcription termination signal, TTTTTTTTT. The strategy for analysis of termination entails the assembly and purification of elongation complexes containing [^{32}P]CMP-labeled 21-mer nascent RNA chains (Fig. 7, lane Pulse). Upon provision of unlabeled nucleoside triphosphates (NTPs) and magnesium, the 3′-*O*-methyl GMP moiety is hydrolyzed by the RNA polymerase (8) and the complexes resume elongation to the end of the linear template; this was evinced by the conversion of the labeled 21-mer RNA into a 195-nucleotide runoff transcript (Fig. 7, lane Chase). The addition of wild-type capping enzyme to the purified transcription complexes just prior to the start of the NTP chase resulted in the appearance of a heterogeneous array of transcripts terminated downstream of the termination signal (Fig. 7). We found that every one of the mutant capping enzymes retained VTF activity (Fig. 7). Apparently, the conserved guanylyltransferase motifs are not constituents of the putative VTF domain of the capping enzyme.

A fraction of the pulse-labeled 21-mer RNAs were not extended during the chase; these represent chains that were released spontaneously from the transcription complexes (7). These released chains could be capped during the chase reaction (which included 1 mM GTP) to form a new labeled species that migrated just above the 21-mer (Fig. 7). No such RNA was formed in the absence of added capping enzyme (compare lanes Chase and WT). Eight of the nine Ala mutants of capping enzyme also formed the guanylylated RNA. The observation that mutant enzymes found to be defective in the standard guanylyltransferase assay (performed in the presence of excess RNA) could cap the released 21-mer transcription product in this experiment can be explained by the fact that the amount of capping enzyme added to the transcription termination reaction mixtures was >100-fold in excess over the amount of labeled nascent RNA. The finding that the D400A mutant did not cap the 21-mer under these conditions (Fig. 7) merely underscored the magnitude of the effect of this mutation on the transguanylylation reaction.

DISCUSSION

We had hypothesized that sequence conservation between mRNA capping enzymes from diverse sources would be rele-

vant to their common mechanism of covalent GMP transfer (32, 36). The present mutational analysis of the vaccinia virus capping enzyme supports this view. Nine conserved amino acids in motifs II to V were targeted for mutagenesis. Six of the nine Ala mutations had a deleterious effect on one or more steps in the guanylyltransferase reaction. Two other mutations rendered the guanylyltransferase thermolabile in vitro. Characterization of the mutant proteins revealed a spectrum of biochemical defects, which are discussed below.

Effects on EpG formation. We have encountered mutational effects on the GTP concentration dependence of EpG formation and the rate of approach to equilibrium in EpG formation. GTP-binding affinity was reduced profoundly by mutations at Glu-375 in motif IV and Lys-392 and Asp-400 in motif V. What role might these residues play as constituents of an NTP-binding site? It is tempting to speculate that Lys-392 may interact directly with the negatively charged phosphates of GTP while either of the acidic residues might coordinate the essential divalent cation cofactor. Alternatively, these residues may interact with the nucleotide base. Given that Glu-375 and Lys-392 are conserved in both GTP-dependent capping enzymes and the ATP-dependent ligases, we favor the idea that these two amino acids interact with the triphosphate moiety. In contrast, Asp-400 is uniquely conserved in motif V of the capping-enzyme family but not in the ligases and may thus be a candidate determinant of nucleotide base specificity.

Mutations at Glu-375 and Lys-392 caused a marked decrease in the rate of the chemical step. At saturating GTP levels, the rate of approach to equilibrium should directly reflect the rate of nucleophilic attack by Lys-260 on the enzyme-bound nucleotide. A full accounting for the rate effect ultimately requires that reaction kinetics be determined for the wild-type and mutant proteins as a function of GTP concentration. (As discussed above, the speed of the wild-type reaction makes this technically difficult; hence, our assays of GTP dependence have dealt solely with reaction yield.) This notwithstanding, we suspect that the kinetic effects of the Glu-375 and Lys-392 mutations seen at 10 μM GTP probably do reflect a true defect in catalysis. How might residues Glu-375 and Lys-392 affect the rate of reaction chemistry? We speculate that they may serve as general base and general acid, respectively. For example, attack on the α-phosphate of GTP requires that the ε-amino group of the active-site lysine be in the unprotonated state. Glu-375 could act as a proton acceptor at this stage. General acid catalysis can be invoked for the expulsion of the PP_i leaving group, in which case Lys-392 could serve as the proton donor.

Several of the mutant proteins (e.g., G376A and T398A) had seemingly wild-type affinity for GTP and near-wild-type kinetics, yet their yield of EpG was lower than that of the wild-type protein, even when normalized to specific ATPase activity. On the other hand, V305A achieved a higher yield than the wild-type enzyme did. The maximum yield of EpG in vitro should represent the absolute value of active enzyme molecules. This is the case provided that all active sites are unoccupied. We have noted that in multiple preparations of capping enzyme from bacteria, the molar yield of EpG formed in vitro is typically 20 to 40% of the theoretical value expected from protein determination (15, 28). This contrasts with capping enzyme purified from virus particles, where EpG formation in vitro is nearly quantitative relative to protein (30). The purified virion particle is impermeable to nucleotides, which may explain why the active site is unoccupied in native enzyme. However, recombinant protein is isolated from bacterial extracts, and it is quite likely that a significant fraction of the enzyme is already guanylylated and remains so during purification in the presence of EDTA. (This is similar to the case of DNA ligase, for which an appreciable fraction of the purified enzyme is already adenylylated.) The present data do not address whether the differences in yield of EpG among the wild-type and mutant capping enzymes are attributable to differences in the extent of preguanylylation. This issue does not affect our conclusions regarding the mutational effects on GTP-binding affinity, catalytic rate, or overall RNA capping (the last of these being a multiple-turnover reaction performed in the presence of excess RNA).

Effects on GMP transfer to RNA. Six of nine Ala mutations affected the transfer of GMP to RNA. Our experiments do not directly address which step in the reaction scheme is affected, i.e., RNA binding to EpG, attack by the 5′ β-phosphate of the cap acceptor on the GMP phosphoramidate, or dissociation of capped RNA product from the enzyme. It is conceivable that mutations affecting a specific step in EpG formation would affect the same step in the RNA guanylylation reaction. For example, residues that interact with the β- or γ-phosphates during GTP binding might make the same contacts with the 5′-diphosphate end of the RNA cap acceptor. Similarly, general acid catalysis is likely to occur during the RNA capping reaction, e.g., by proton donation to the Lys-260 leaving group. However, although the chemistry of the RNA transfer step is similar to that of EpG formation, it is quite possible, indeed likely, that there are protein-substrate contacts and conformational steps that are specific to the RNA transfer event.

Perhaps the most interesting mutant in our collection is E303A, which has little discernible affect on EpG formation but selectively reduces GMP transfer to RNA. Early studies of the vaccinia virus capping enzyme made clear that efficient utilization of cap acceptors requires both a 5′-diphosphate end and a polynucleotide chain; the K_m for mononucleoside diphosphate is about 40-fold higher than the K_m for diphosphate-terminated polynucleotide (18, 19). A plausible explanation for the mutational selectivity is that Glu-303 is involved in binding to the RNA cap acceptor at a site other than the terminal phosphates.

Domain specificity. The triphosphatase and guanylyltransferase domains both reside within the N-terminal segment of the D1 subunit. To what extent do the two functional units overlap structurally? Our first indication that the active site of γ-phosphate cleavage is distinct from that of nucleotidyl transfer was our finding that a mutation at Lys-260 (which completely abrogated EpG formation) had no effect on the triphosphatase (3). The present study indicates that residues in motifs III, IV, and V that are important for transguanylylation are not particularly relevant to the triphosphatase reaction. The emerging theme is that the two reactions are mediated by distinct structural elements. Similar conclusions can be drawn about the transcription termination factor activity of the vaccinia virus capping enzyme. Mutations at the guanylyltransferase active site (15) or within motifs II to IV were well tolerated with respect to VTF activity. As yet, there has been no report of any amino acid substitution mutations of the capping enzyme that selectively affect the triphosphatase or VTF components.

None of the mutations in motifs II to V inhibited the methyltransferase; this was not surprising, given that cap methylation is catalyzed by a structural unit separated from the guanylyltransferase by an interdomain hinge. The methyltransferase specific activity of the K392A mutant was conspicuously higher than that of the wild-type capping enzyme even after the values were normalized to the ATPase activity. The substrate for the methylation reaction is cap-labeled poly(A), which, in principle, can bind either to the methyl acceptor site located within

the C-terminal portion of the D1 subunit or to the product RNA-binding site of the guanylyltransferase domain in the N-terminal portion of the D1 protein. An increase in apparent methyltransferase activity could arise if the K392A mutation disrupts the RNA-binding site of the guanylyltransferase (as suggested above), indirectly allowing the substrate to partition in favor of the methyl acceptor site.

Comparison of mutational effects on vaccinia virus and yeast capping enzymes. The *S. cerevisiae* capping enzyme is a 459-amino-acid polypeptide encoded by the essential *CEG1* gene (26). Unlike the vaccinia virus D1 subunit, which is clearly multifunctional, the yeast protein has no known activity other than guanylyltransferase. Parallel mutational analysis of the yeast capping enzyme indicated that motifs III, IV, and V were essential for CEG1 function in vivo (32). It is satisfying to note that the in vitro effects of mutations in the vaccinia virus D1 protein are entirely consistent with the in vivo consequences of Ala substitutions at homologous positions in CEG1.

Mutation of the Glu residue in motif III of CEG1 was lethal in vivo (32); the corresponding E303A mutation of vaccinia virus D1 caused a specific block in the second GMP transfer reaction. This acidic residue is conserved in all capping enzymes and in all members of the ATP-dependent ligase family aligned in Fig. 1. Indeed, we have recently found that substitution of this Glu residue by Ala inhibited the DNA strand-joining activity of vaccinia virus DNA ligase (35). This underscores the value of the motif alignment in predicting relevant structure-function relationships. The neighboring Gly residue in motif III is not strictly essential for capping-enzyme function in *S. cerevisiae* or vaccinia virus in that it can be replaced by Ala, a relatively conservative change. This is perhaps not surprising, given that some members of the ligase family contain alternative residues at this position. However, Ala substitution at the Gly residue was not entirely without effect. It caused a thermosensitive growth defect in *S. cerevisiae* and rendered the vaccinia virus guanylyltransferase temperature sensitive in vitro. The V305A mutation in motif III had no discernible effect on vaccinia virus capping enzyme, and the equivalent mutant with a mutation in yeast CEG1 was fully viable (32).

Mutations at the conserved acidic and Gly residues in motif IV were lethal in *S. cerevisiae*, just as they severely affected RNA capping in vitro by the vaccinia virus enzyme. (Similarly, Ala substitution for Glu in motif IV of vaccinia virus ligase abolished DNA strand joining [35].) The conserved Lys and Asp positions in motif V were required for in vitro activity of the vaccinia virus capping enzyme and were also essential for CEG1 function in vivo. Mutation of the invariant Thr residue in motif V of vaccinia virus D1 partially inactivated both nucleotidyl transfer steps in vitro; the same mutation in CEG1 caused a temperature-sensitive growth defect.

Motif II is apparently not critical for activity of either the *S. cerevisiae* or vaccinia virus capping enzyme. In retrospect, this is not surprising, given that this motif is not found in all capping enzymes or in most of the ligases. The temperature sensitivity of motif II mutants, either in vivo or in vitro, suggests that motif II may play a role in maintaining protein structure rather than in catalysis.

Conclusions. We have shown that residues within conserved motifs III, IV, and V are important for nucleotidyl transfer by vaccinia virus capping enzyme. Our findings illuminate for the first time the distinctive roles of individual residues at specific steps of the capping reaction. It is likely that these findings will apply to other capping enzymes and to the polynucleotide ligases. A fully quantitative treatment of the mutational effects on the vaccinia virus capping enzyme was not attempted; this must await complete kinetic analysis of the wild-type enzyme, including determination of the individual rate constants for all steps in the reaction pathway. A definitive assignment of function to individual residues and individual reaction steps will ultimately require a crystal structure of a member of the guanylyltransferase (or ligase) family, ideally with bound nucleotide donor or nucleic acid acceptor. In the absence of a crystal structure, we suggest that further mutational analyses targeted to regions of conservation between the capping enzymes and ligases will provide useful structure-function insights for both enzyme families.

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