

Yeast-Based Genetic System for Functional Analysis of Poxvirus mRNA Cap Methyltransferase

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Structural differences between poxvirus and human mRNA capping enzymes recommend cap formation as a target for antipoxviral drug discovery. Genetic and pharmacologic analysis of the poxvirus capping enzymes requires in vivo assays in which the readout depends on the capacity of the viral enzyme to catalyze cap synthesis. Here we have used the budding yeast *Saccharomyces cerevisiae* as a genetic model for the study of poxvirus cap guanine-N7 methyltransferase. The *S. cerevisiae* capping system consists of separate triphosphatase (Cet1), guanylyltransferase (Ceg1), and methyltransferase (Abd1) components. All three activities are essential for cell growth. We report that the methyltransferase domain of vaccinia virus capping enzyme (composed of catalytic vD1-C and stimulatory vD12 subunits) can function in lieu of yeast Abd1. Coexpression of both vaccinia virus subunits is required for complementation of the growth of *abd1Δ* cells. Previously described mutations of vD1-C and vD12 that eliminate or reduce methyltransferase activity in vitro either abolish *abd1Δ* complementation or elicit conditional growth defects. We have used the yeast complementation assay as the primary screen in a new round of alanine scanning of the catalytic subunit. We thereby identified several new amino acids that are critical for cap methylation activity in vivo. Studies of recombinant proteins show that the lethal vD1-C mutations do not preclude heterodimerization with vD12 but either eliminate or reduce cap methyltransferase activity in vitro.

The m⁷GpppN cap structure of eukaryotic mRNAs is formed cotranscriptionally by the sequential action of three enzymes: an RNA triphosphatase that removes the gamma phosphate of the nascent transcript to yield a 5' diphosphate end, a GTP:RNA guanylyltransferase that adds a GMP residue to the diphosphate end in a 5'-to-5' orientation, and an RNA (guanine-N7) methyltransferase that uses S-adenosylmethionine to methylate the N7 position of the cap guanine (34, 35). The capping enzyme of vaccinia virus is a heterodimer of vD1 (844 amino acids [aa]) and vD12 (287 aa) polypeptides that catalyzes all three capping reactions (24, 38). The triphosphatase, guanylyltransferase, and methyltransferase active sites are arrayed in modular fashion within the vD1 subunit. The amino-terminal 60-kDa segment of vD1 (from aa 1 to 545) is an autonomous triphosphatase-guanylyltransferase domain in which the triphosphatase active site is located in the proximal half and the guanylyltransferase active site is in the distal half (8, 9, 12, 26, 27, 28, 37, 41, 42). The methyltransferase domain consists of the carboxyl-terminal portion of the vD1 subunit (vD1-C) heterodimerized with the vD12 subunit (7, 15, 16, 17, 20, 21, 29). The methyltransferase active site is located within vD1-C, which has a weak intrinsic methyltransferase activity that is stimulated 30- to 50-fold by the vD12 subunit, which is itself catalytically inert. Thus, the native vaccinia virus cap methyltransferase consists of a catalytic subunit (vD1-C) and a regulatory subunit (vD12).

The physical organization of the poxvirus capping apparatus

is quite distinct from that of cellular capping enzymes (34, 35). For example, the triphosphatase, guanylyltransferase, and methyltransferase components of the yeast capping apparatus reside on three different polypeptides encoded by three genes (*CET1*, *CEG1*, and *ABD1*, respectively). The metazoan capping enzyme consists of two components, a bifunctional triphosphatase-guanylyltransferase polypeptide and a separately encoded methyltransferase polypeptide. The cap methyltransferases of fungi and metazoa are monomeric enzymes. In the budding yeast *Saccharomyces cerevisiae*, the cap methylating enzyme Abd1 is essential for cell viability (22, 23, 40). Cap methyltransferases from other unicellular eukarya (*Ccm1*, *Pcm1*, and *Ecm1* of *Candida albicans*, *Schizosaccharomyces pombe*, and *Encephalitozoon cuniculi*, respectively) can function in lieu of Abd1 to sustain yeast cell growth (14, 30). The human cap methyltransferase Hcm1 can also replace Abd1 in vivo, attesting to the conservation of structure and function between cap methylating enzymes from yeast to humans (30). Cellular cap methyltransferases display local amino acid sequence similarity to the vaccinia virus methyltransferase catalytic subunit but not to the stimulatory subunit (40).

The structural differences between the poxvirus and human capping enzymes recommend cap formation as a target for antipoxviral drug discovery. The stimulatory subunit of the viral methyltransferase is an especially attractive molecular target, because there is no discernible homolog of vD12 in the known proteomes of any metazoan species. Although structure-function analyses of vaccinia virus cap methyltransferase have been carried out with purified vD1-C and D12 proteins expressed in bacteria, there has been no genetic analysis of the role of cap N7 methylation in poxvirus replication in vivo. Mutations that elicit temperature-sensitive virus growth phe-

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notypes have been mapped to the two capping enzyme subunits (6, 13), but the picture is complicated because the capping enzyme plays multiple additional roles in viral gene expression. Capping enzyme serves as a transcription termination factor during the synthesis of viral early mRNAs (19, 36) and as an initiation factor during the transcription of intermediate genes (39). Surprisingly, the vD1 and vD12 temperature-sensitive mutant viruses display no gross defect in viral gene expression at the restrictive temperature but instead are defective in resolving concatameric DNA replication intermediates into the hairpin telomeres of the mature viral genome (6, 13). The telomere phenotype has no obvious connection to the known mRNA processing functions of the vD1 and vD12 proteins.

Genetic and pharmacologic studies of poxvirus capping enzymes would be expedited by the development of *in vivo* assays in which the functional readout is clearly and exclusively dependent on the capacity of the viral gene product to catalyze cap synthesis. Previously, Ho et al. validated the concept of using the budding yeast *S. cerevisiae* as a genetic model for the study of the RNA triphosphatase and guanylyltransferase components of the poxvirus capping enzyme (18). Ho et al. showed that expression of vD1(1-545) in *S. cerevisiae* complemented the growth of mutant strains lacking the endogenous RNA triphosphatase or guanylyltransferase enzymes provided that vD1(1-545) was fused to a cellular protein capable of targeting it to the RNA polymerase II (Pol II) transcription complex.

Here we tested the feasibility of using yeast as a genetic system to study the function of the poxvirus cap methyltransferase. We report that the vaccinia virus methyltransferase functions in lieu of the yeast Abd1 when the vD1-C and vD12 proteins are coexpressed *in vivo*. Genetic complementation by the viral methyltransferase did not require fusion to a cellular targeting protein. We exploit the yeast-based genetic system to identify constituents of the poxvirus catalytic and regulatory subunits that are important for cap methylation *in vivo* and *in vitro*.

MATERIALS AND METHODS

Yeast expression plasmids for vaccinia virus cap methyltransferase. The open reading frame encoding the methyltransferase catalytic subunit vD1-C (aa 498 to 844) was PCR amplified from pET14-His-D1(498 to 844) (20). The PCR product was inserted into yeast expression vector pYN132 (*CEN TRP1*) such that expression of vD1-C was under the control of the yeast *TPII* promoter. The *TPII-vD1-C* cassette was then inserted into a multicopy 2 μ m *TRP1* vector. *CEN TRP1* and 2 μ m *TRP1* plasmids carrying mutated versions of vD1-C (*H682A-Y683A*, *D598A*, *G600A*, *G602A*, *Y683S*, *S684A*, and *F685A*) were constructed similarly using the respective mutant pET14-D1 (aa 498 to 844) plasmids (20) as templates for PCR amplification. Other vD1-C mutants were generated via the two-stage PCR overlap extension method and then cloned into yeast *CEN* and 2 μ m expression vectors. All DNA inserts were sequenced completely to ensure that no unwanted coding mutations were introduced during amplification and cloning.

The vD12 gene encoding the stimulatory subunit of the vaccinia virus cap methyltransferase was excised from pET16-D12 (31) by digestion with *NdeI* and *BamHI* and then inserted into pYN132. The expression cassette *TPII-vD12* was then transferred into plasmids pRS413 (*CEN HIS3*) and pRS423 (2 μ m *HIS3*). The same strategy was employed to generate the plasmids for expression in yeast of the mutant vD12 proteins N42A-Y43A, L61A-K62A, F176A-K177A, and F245A-L246A.

Plasmid shuffle assay for cap methyltransferase activity *in vivo*. *S. cerevisiae* strain YBS40 lacks the chromosomal *ABD1* locus encoding the yeast cap methyltransferase (30). Growth of YBS40 depends on the maintenance of plasmid p360-ABD1(*CEN URA3 ABD1*). YBS40 was cotransformed with yeast expression plasmids carrying wild-type or mutant vD1-C and vD12. Trp⁺His⁺ trans-

formants were selected at 30°C on agar medium lacking tryptophan and histidine. Cells were then streaked at 37, 30, 25, and 19°C on agar medium containing 0.75 mg of 5-FOA/ml. The plates were incubated at 37, 30, 25, and 19°C for up to 8 days. The lethal alleles were those that failed to yield colonies on 5-FOA at any temperature. Individual FOA-resistant colonies arising at permissive temperature were patched to yeast extract-peptone-dextrose (YEPD) agar. Cells were then streaked on YEPD agar, and growth was scored at different temperatures.

Coexpression of vD1-C and vD12 proteins in bacteria and enzyme purification. The wild-type vD12 gene was inserted into pET3c to yield pET3-D12. The wild-type vD1-C open reading frame was cloned into pET16b. *NdeI-XhoI* fragments encoding mutated versions of vD1-C were excised from the respective pYN132-vD1-C constructs and transferred into the pET16b vector. To generate coexpression plasmids, the expression cassettes for wild-type vD1-C and the alanine mutants were excised from the pET16b plasmids with *BglII* and *HindIII* and then inserted between the *BamHI* and *HindIII* sites of pET3-D12. Transcription of the vD1-C and vD12 genes in the coexpression vector was driven by separate tandemly oriented T7 promoters. The plasmids were transformed into *Escherichia coli* BL21(DE3).

E. coli BL21(DE3)/pET transformants were inoculated into Luria-Bertani medium containing 0.1 mg of ampicillin/ml and grown at 37°C until the A_{600} reached 0.3. The cultures (500 ml) were placed on ice for 30 min, adjusted to either 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside) [for His-vD1-C/D12, His-vD1-C(R682A)/vD12, His-D1-C(F780A)/vD12, and His-D1-C(F814A-F815A)/vD12 expression] or 0.15 mM IPTG (for all others). The cultures were incubated for 18 to 20 h at 17°C with continuous shaking. Cells were harvested by centrifugation, and the pellets were stored at -75°C. All subsequent procedures were performed at 4°C.

Cell pellets were thawed on ice and suspended in 25 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 10% glycerol). Cell lysis was achieved by addition of lysozyme and Triton X-100 to achieve final concentrations of 0.25 mg/ml and 0.1%, respectively. Insoluble material was removed by centrifugation at 17,000 rpm for 30 min in a Sorvall SS34 rotor. The supernatants were mixed for 1 h with 2.5 ml of Ni-nitrilotriacetic acid-agarose resin (Qiagen) that had been equilibrated with lysis buffer. The slurries were poured into columns and then washed with lysis buffer. The columns were eluted stepwise with IMAC buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 10% glycerol, 0.1% Triton X-100) containing 50, 200, and 500 mM imidazole. The polypeptide compositions of the fractions were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant vaccinia virus proteins were recovered in the 200 mM imidazole eluate. The concentrations of His-vD1-C and vD12 proteins were determined by SDS-PAGE analysis of protein samples in parallel with 0.5, 1.0, 1.5, and 2.0 μ g of bovine serum albumin. The gels were fixed and stained with Coomassie blue dye. The staining intensities of the polypeptides were quantified using a Digital Imaging and Analysis system (Alpha Innotech Corporation). The concentrations of the wild-type and the mutant D1 proteins were calculated by interpolation to the bovine serum albumin standard curve.

Methyltransferase assay. Protein samples were adjusted with 50 mM Tris-HCl (pH 7.5)-5 mM dithiothreitol to contain ~20 fmol of the vD1-C subunit per μ l. Aliquots (2 μ l) of the protein solutions and serial dilutions thereof were assayed for cap methylation in reaction mixtures (10 μ l) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 50 μ M AdoMet, and 15 fmol of ³²P cap-labeled poly(A) RNA (33). The mixtures were incubated at 37°C for 5 min. The reactions were quenched by heating to 95°C for 5 min. The mixtures were adjusted to 50 mM sodium acetate (pH 5.5) and then digested with 5 μ g of nuclease P1 for 1 h at 37°C. The digests were spotted on polyethyleneimine cellulose thin-layer chromatography plates that were developed with 0.35 M (NH₄)₂SO₄. The extent of methylation of the cap [expressed as m⁷GpppA/(m⁷GpppA+GpppA)] was determined by scanning the chromatogram with a phosphorimager.

RESULTS

Vaccinia virus cap methyltransferase is active in yeast. We used the plasmid shuffle technique to test the capacity of the vaccinia virus cap methyltransferase to complement the *abd1Δ* mutation of *S. cerevisiae* (Fig. 1). *abd1Δ* cells containing a copy of *ABD1* on a *CEN URA3* plasmid were cotransformed with vD1-C (2 μ m *TRP1*) and vD12 (2 μ m *TRP1*) expression plasmids. *ABD1* (*CEN TRP1*) served as a positive control. Transformants were selected and then tested for growth on medium

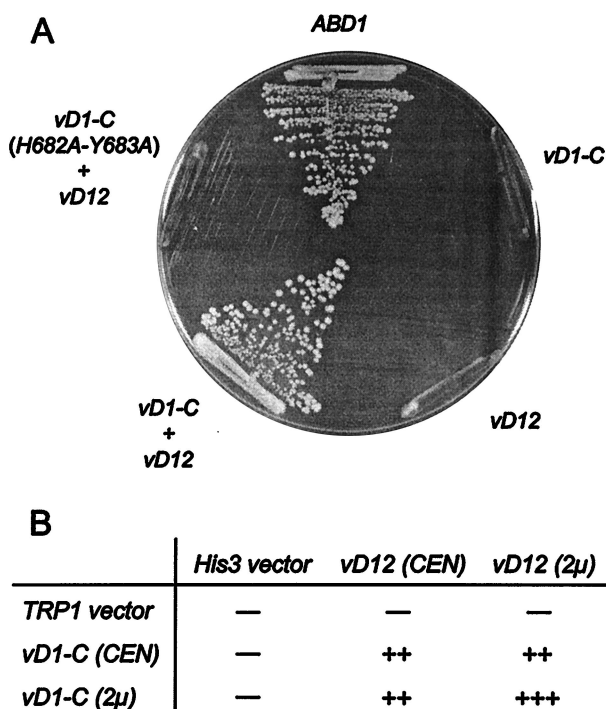


FIG. 1. Complementation of *abd1* Δ cells by vaccinia virus cap methyltransferase. (A) Growth on 5-FOA medium. Yeast strain YBS40 (*abd1* Δ) was transformed with 2 μ m plasmids containing either *vD1-C* (*TRP1*), *vD12* (*HIS3*), *vD1-C* plus *vD12*, or *vD1-C* (*H682A-Y683A*) plus *vD12*. A control transformation was performed with a *CEN TRP1* plasmid containing the *ABD1* gene. Single transformants were streaked to medium containing 5-FOA. The plate was photographed after incubation for 4 days at 30°C. (B) *vD1-C* and *vD12* were introduced into YBS40 cells on *CEN* and 2 μ m plasmids in combinations as indicated and were tested by plasmid shuffle. Strains that failed to grow on 5-FOA are indicated by minuses. The growth of 5-FOA survivors was assessed on rich medium and recorded after 2 days of incubation at 30°C. +++ indicates that the colony size was indistinguishable from that of *ABD1* cells, and ++ indicates that the strain formed slightly smaller colonies than wild-type cells.

containing 5-fluoroorotic acid (5-FOA), a drug that selects against the *URA3 ABD1* plasmid. Transformants that contained both *vD1-C* and *vD12* on 2 μ m plasmids readily formed 5-FOA-resistant colonies, but cells transformed singly with either *vD1-C* or *vD12* on a 2 μ m plasmid did not (Fig. 1). Thus, the heterodimeric vaccinia virus methyltransferase can function in yeast and both subunits are necessary for cap methylation in vivo.

To assess the influence of gene dosage on in vivo methyltransferase activity, *abd1* Δ cells were transformed with various combinations of *vD1-C* and *vD12* on either multicopy 2 μ m or single-copy *CEN* plasmids. All combinations yielded 5-FOA-resistant strains, which were then tested for growth on rich (YEPD) agar medium. Cells expressing *vD1-C* and *vD12* on 2 μ m plasmids grew as well as wild-type *ABD1* cells (Fig. 1B), whereas cells carrying either *vD1-C* or *vD12* on a *CEN* plasmid formed slightly smaller colonies.

Vaccinia virus methyltransferase activity is essential for *abd1* Δ complementation. The *vD1-C* mutation *H682A-Y683A* abolishes methyltransferase activity in vitro without affecting

TABLE 1. Effects of biochemically characterized *vD1-C* mutations on cap methyltransferase function in vivo^a

<i>vD1-C</i> allele + <i>vD12</i>	Growth at temp (°C): ^b		
	19	30	37
<i>CEN</i>			
WT ^c	++	++	++
D598A	+	—	—
G600A	—	—	—
G602A	+	+	+
Y683S	—	—	—
S684A	++	++	+
F685A	++	++	+
2 μ m			
WT	+++	+++	+++
D598A	+	+	—
G600A	—	—	—
G602A	+++	++	++
Y683S	—	—	—
S684A	+++	++	++
F685A	+++	++	++

^a The indicated alleles of *vD1-C* were tested by plasmid shuffle for complementation of *abd1* Δ as described in Methods and Materials. *CEN vD1-C* plasmids were cotransformed with *CEN vD12* (*CEN*), and 2 μ m *vD1-C* plasmids were cotransformed with 2 μ m *vD12* (2 μ m).

^b +++ indicates colony sizes indistinguishable from those of strains bearing wild-type *ABD1*, ++ indicates slightly reduced colony sizes, + indicates that only pinpoint colonies were formed, and — (minus) indicates no growth.

^c WT, wild type.

subunit heterodimerization (21). Here we found that a *vD1-C* (*H682A-Y683A*) mutant on a 2 μ m plasmid failed to complement *abd1* Δ when cotransformed with *vD12* (Fig. 1A). This result indicates that methyltransferase catalytic activity is essential for complementation of *abd1* Δ by the poxvirus capping enzyme.

Six other previously characterized *vD1-C* mutants that retained the capacity to heterodimerize with *vD12* were also tested for complementation of *abd1* Δ . *CEN* and 2 μ m plasmids expressing the *vD1-C* mutants were introduced into *abd1* Δ cells along with *CEN* or 2 μ m plasmids expressing wild-type *vD12* (Table 1). Transformants were selected and streaked on 5-FOA agar plates at 19, 30, and 37°C. *vD1-C* alleles *G600A* and *Y683S* were lethal at all temperatures, whether on single-copy or multicopy plasmids. We surmise that lethality is a consequence of the defect in catalyzing cap methylation by the mutant proteins, insofar as the purified recombinant *G600A/vD12* and *Y683S/vD12* heterodimers exhibited 4% and 0.1% of wild-type methyltransferase activity in vitro, respectively (21).

D598A supported the emergence of 5-FOA-resistant colonies only at low temperatures. Cells carrying the *vD1-C-D598A* mutant on a single-copy plasmid formed pinpoint colonies at 19°C but failed to grow above this temperature. Increasing the copy number of the *D598A* and *vD12* genes allowed formation of pinpoint colonies at 19 and 30°C but no growth at 37°C (Table 1). It was reported previously that the purified *D598A/vD12* heterodimer was 50% as active as the wild-type vaccinia virus methyltransferase in vitro (21). Thus, the conditional growth phenotype may reflect the decrement in methyltransferase catalytic activity.

5-FOA-selected cells containing *G602A* on a *CEN* plasmid formed pinpoint colonies on YEPD medium at 19, 30, and

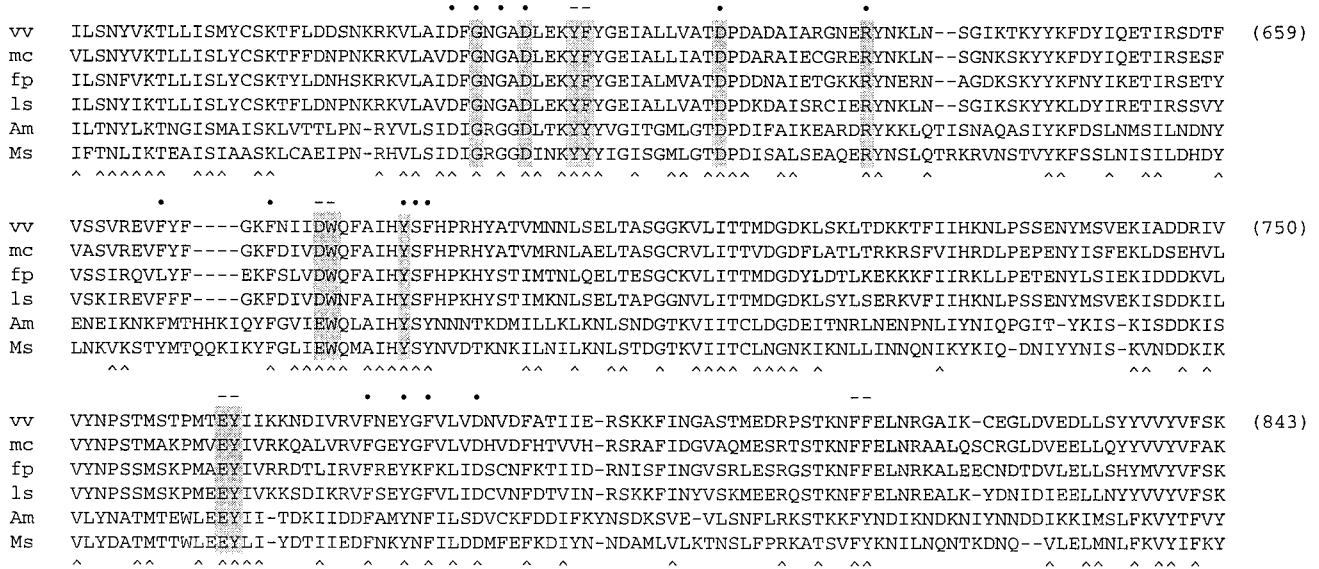


FIG. 2. Sequence alignment of poxvirus vD1-C orthologs. The amino acid sequence of vaccinia virus D1 (vv) from aa 573 to 843 was aligned with the sequences of orthologous polypeptides of molluscum contagiosum virus (mc), fowlpox virus (fp), lumpy skin disease virus (ls), *Amsacta moorei* entomopoxvirus (Am), and *Melanoplus sanguinipes* entomopoxvirus (Ms). Positions of side chains with identity or similarity in all six poxvirus proteins are indicated by a caret below the aligned sequences. Missense mutations at positions (marked by ●) and double-alanine mutations (indicated by double dashes) were tested for in vivo function in yeast. The conserved amino acids at which mutations resulted in unconditional lethality in yeast are highlighted by shading.

37°C (Table 1). Increasing the dosage of the *G602A* and *vD12* genes improved growth at all temperatures. The size of the 2µm *G602A/vD12* colonies at 19°C was indistinguishable from that of “wild-type” 2µm *vD1-C/vD12* cells; the colony size of the mutant strain was slightly smaller than that of the wild-type strain at 30 and 37°C (Table 1). Earlier studies showed that the methyltransferase activity of the purified *G602A/vD12* heterodimer was identical to that of wild-type vaccinia virus methyltransferase (21). The present finding that *vD1-C-G602A/vD12* cells displayed a severe slow growth phenotype that was suppressed by overexpression of both components of the cap methyltransferase suggests that (i) the *G602A* mutation can affect protein folding or stability when vD1-C is expressed in yeast or (ii) the *G602A* mutation can reduce the affinity of vD1-C for the vD12 subunit in yeast.

S684A and *F685A* strains expressing the methyltransferase subunits from *CEN* plasmids grew as well as wild-type *vD1-C/vD12* cells at 19 and 30°C but formed smaller colonies at 37°C (Table 1). Increasing the dosage of the *S684A* and *F685A* genes resulted in improved growth at both 19 and 37°C. These results are consistent with biochemical studies showing that purified recombinant *S684A/vD12* and *F685A/vD12* heterodimers exhibited 60 to 80% of wild-type methyltransferase activity in vitro (21).

Genetic screen for new mutations affecting cap methyltransferase function in vivo. Our initial genetic analysis of biochemically defined mutants of vaccinia virus cap methyltransferase indicated that (i) lesions in the catalytic subunit that abolish activity in vitro were consistently lethal in vivo and (ii) the yeast complementation assay was sensitive to mutations resulting in partial loss of cap methyltransferase activity which resulted in conditional phenotypes and/or growth defects that were sup-

pressible by increased gene dosage. We therefore sought to exploit the yeast-based genetic assay as a primary screening method for identification of new structural components of the catalytic subunit that contribute to methyltransferase function.

We used the plasmid shuffle procedure to test the effects of single-alanine substitutions in vD1-C at positions Asp604, Asp620, and Arg632, which are conserved in all poxvirus D1 orthologs (Fig. 2) as well as in cellular cap methyltransferases (4, 32, 40). The *vD1-C* alleles *D604A*, *D620A*, and *R632A* were lethal at all temperatures, whether on single-copy or multicopy plasmids (Table 2). These findings are concordant with previous studies showing that the equivalent Asp and Arg chains were essential for the in vivo activity of yeast cap methyltransferase Abd1 and human cap methyltransferase Hcm1 (23, 30, 32, 40).

We then tested the effects of single-alanine substitutions at five aromatic residues (Phe667, Phe672, Phe775, Tyr778, and Phe780) and one acidic residue (Asp784) that are conserved in other poxvirus capping enzymes (Fig. 2). We focused on aromatic residues in light of crystallographic findings for several proteins involved in cap formation or cap recognition, which indicate that the protein-bound cap guanine is stabilized by π-stacking interactions with aromatic amino acids (5). We found that FOA-selected cells containing either the *F667A*, *F672A*, or *Y778A* alleles on *CEN* plasmids grew as well as wild-type vD1-C/vD12 cells on rich medium at all temperatures tested (Table 2). We therefore concluded that the Phe667, Phe672, and Tyr778 side chains were not important for methyltransferase activity in vivo.

In contrast, the *F775A* mutation elicited a growth defect at all temperatures when present in single copy. However, the growth phenotype was suppressed completely at all tempera-

TABLE 2. New alanine-scanning mutagenesis of vD1-C^a

vD1-C allele + vD12	Growth at temp (°C): ^b		
	19	30	37
<i>CEN</i>			
WT ^c	++	++	++
D604A	-	-	-
Y608A-F609A	-	-	-
D620A	-	-	-
R632A	-	-	-
F667A	++	++	++
F672A	++	++	++
D676A-W677A	-	-	-
E763A-Y764A	-	-	-
F775A	+	+	+
Y778A	++	++	++
F780A	+	+	-
D784A	+	+	+
F814A-F815A	-	-	-
<i>2μm</i>			
WT	+++	+++	+++
D604A	-	-	-
Y608A-F609A	-	-	-
D620A	-	-	-
R632A	-	-	-
D676A-W677A	-	-	-
E763A-Y764A	-	-	-
F775A	+++	+++	+++
F780A	++	++	++
D784A	+++	+++	+
F814A-F815A	+	+	+

^a The indicated alleles of vD1-C were tested by plasmid shuffle for complementation of *abd1Δ*. *CEN* vD1-C plasmids were cotransformed with *CEN* vD12 (*CEN*), and 2μm vD1-C plasmids were cotransformed with 2μm vD12 (2μm).

^b +++ indicates colony sizes indistinguishable from those of strains bearing wild-type *ABD1*, ++ indicates slightly reduced colony sizes, + indicates that only pinpoint colonies were formed, and - indicates no growth.

^c WT, wild type.

tures by increased gene dosage (Table 2). Similarly, the *F780A* and *D784A* mutations in single copies resulted in slow growth at 19 and 30°C and no growth at 37°C and these phenotypes were suppressed by increases in gene copy number (Table 2). For example, 2μm *F780A* cells displayed improved growth at permissive temperatures and a gain of viability at 37°C. 2μm *D784A* cells grew as well as wild-type cells at 19 and 30°C, although they remained temperature sensitive, forming pinpoint colonies at 37°C (Table 2). It is noteworthy that three conserved amino acids at which single-alanine mutations conferred dosage-suppressible growth defects (Phe775, Phe780, and Asp784) are clustered together in the vD1-C polypeptide.

Finally, we tested the effects of four new double-alanine mutations in adjacent conserved residues of vD1-C (Fig. 2). The vD1-C alleles *Y608A-F609A*, *D676A-W677A*, and *E763A-Y764A* were lethal at all temperatures, whether on single-copy or multicopy plasmids (Table 2). *F814A-F815A* was lethal in single copy but supported the formation of pinpoint colonies when present in high copy together with 2μm vD12 (Table 2).

Mutational effects on methyltransferase activity in vitro. Eight of the new vD1-C mutants were produced as recombinant proteins in bacteria in parallel with the wild-type protein and subjected to biochemical analysis of subunit heterodimerization and cap methylation activity. The mutants selected for in vitro analysis were either lethal in yeast at high gene dosage

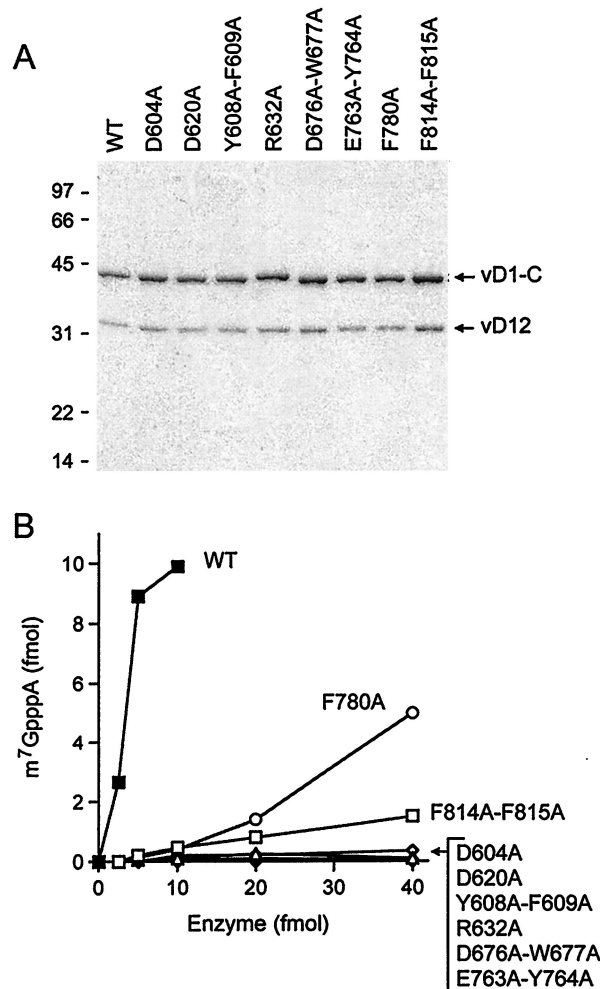


FIG. 3. Mutational effects on cap methyltransferase activity in vitro. (A) Enzyme purification. Aliquots of the Ni-agarose fractions of recombinant His-vD1-C/vD12 (containing 1 μg of the vD1-C subunit) were analyzed by SDS-PAGE. The polypeptides were visualized by staining with Coomassie blue dye. The positions and sizes (in kilodaltons) of marker proteins are indicated at the left. (B) Cap methyltransferase activity. Activity was assayed and quantitated as described in Methods and Materials. The extent of cap methylation is plotted as a function of the amount of recombinant vD1-C included in the reaction mixture.

(*D604A*, *Y608A-F609A*, *D620A*, *R632A*, *D676A-W677A*, and *E763A-Y764A*) or displayed growth defects of various severity at high gene dosage (*F780A* and *F814A-F815A*). The His-tagged mutant vD1-C polypeptides were coexpressed in bacteria with the untagged wild-type vD12 subunit. Purification from soluble bacterial extracts was achieved by Ni-agarose affinity chromatography, which selects for the His-tagged vD1-C subunit. Polypeptides corresponding to vD1-C and vD12 were the predominant species recovered after the affinity chromatography of the wild-type enzyme (Fig. 3A, lane WT). The preparations of His-tagged mutants *D604A*, *Y608A-F609A*, *D620A*, *R632A*, *D676A-W677A*, *E763A-Y764A*, *F780A*, and *F814A-F815A* also contained the vD12 subunit. The extents of purification were comparable for the wild-type

and mutant methyltransferases, and the ratios of the vD1-C and vD12 subunits were similar for the wild-type and mutant enzymes, as gauged by the results of SDS-PAGE and staining with Coomassie blue dye (Fig. 3A). We conclude that the D1-C-Ala mutations selected for their severe in vivo phenotypes in yeast had no significant effect on vD1-C interaction with vD12 when the vaccinia virus subunits were coexpressed in *E. coli*.

The recombinant proteins were assayed for their ability to catalyze AdoMet-dependent conversion of ^{32}P cap-labeled poly(A) [$\text{GpppA}(\text{pA})_n$] to methylated cap-labeled poly(A) [$\text{m}^7\text{GpppA}(\text{pA})_n$] (boldface characters indicate the labeled phosphorus). The specific activities of the D604A, D620A, Y608A-F609A, D676A-W677A, R632A, and E763A-Y764A enzymes were <1% of the wild-type value (Fig. 3B). Note that these six mutations were lethal in vivo. The F780A and F814A-F815A enzymes displayed low residual methyltransferase activity (approximately 7.1% and 2.2% of the wild-type specific activity, respectively) (Fig. 3B). The growth phenotypes of these two mutants in vivo at high gene dosage (++ for F780A and + for F814A-F815A) correlated with their relative cap methyltransferase activities in vitro. We conclude from these experiments that *abd1* Δ complementation is an effective primary screen for pinpointing essential structural features of the catalytic subunit of the poxvirus cap methyltransferase.

Effects of mutations in the D12 subunit of vaccinia virus cap methyltransferase. Complementation of *abd1* Δ cells by vaccinia virus cap methyltransferase is dependent on the stimulatory vD12 subunit (Fig. 1). Thus, the genetic assay should be sensitive to mutations of the vD12 subunit that either diminish its affinity for the catalytic subunit or affect its allosteric activation. Saha and Shuman previously identified and characterized double-alanine mutations of adjacent amino acids of vD12 that compromised its interaction in vitro with vD1-C (31). Here we tested the effects of such mutations on the in vivo activity of the vaccinia virus methyltransferase in yeast. vD12 mutants L61A-K62A, F176A-K177A, F245A-L246A, and N42A-Y43A were cloned into *CEN* and $2\mu\text{m}$ yeast expression vectors, which were then introduced into yeast *abd1* Δ cells together with the wild-type vD1-C gene on a *CEN* or $2\mu\text{m}$ plasmid. Complementation was tested by plasmid shuffle, and 5-FOA survivors were streaked on YEPD agar at 19, 30, and 37°C (Table 3).

F176A-K177A was lethal at all temperatures in single copy and viable but severely defective in high copy, i.e., $2\mu\text{m}$ F176A-K177A cells formed pinpoint colonies at 19 and 30°C and failed to grow at 37°C. The three other vD12 mutants displayed less drastic phenotypes in single copy that were suppressed completely by increased gene dosage (Table 3). Cells bearing L61A-K62A or F245A-L246 in single copy grew as well as wild-type vD1-C cells at 19 and 30°C but were inviable at 37°C. *CEN* N42A-Y43A cells grew as well as wild-type vD1-C cells at 19°C, grew more slowly at 30°C, and failed to form colonies at 37°C. Yet $2\mu\text{m}$ L61A-K62A, F245A-L246, and N42A-Y43A cells displayed +++ growth at all temperatures.

The findings that vD12 mutations that affect subunit heterodimerization in vitro display temperature sensitivity in vivo and dosage suppression suggest that (i) the effects of the vD12 mutations on its affinity for the catalytic subunit are exacerbated at 37°C and (ii) weakened subunit affinity can be com-

TABLE 3. Effects of vD12 mutations on *abd1* complementation^a

vD12 allele + vD1-C	Growth at temp (°C): ^b		
	19	30	37
<i>CEN</i>			
WT ^c	++	++	++
N42A-Y43A	++	+	-
L61A-K62A	++	++	+
F176A-K177A	-	-	-
F245A-L246A	++	++	-
$2\mu\text{m}$			
WT	+++	+++	+++
N42A-Y43A	+++	+++	+++
L61A-K62A	+++	+++	+++
F176A-K177A	+	+	-
F245A-L246A	+++	+++	+++

^a The indicated alleles of vD12 were tested by plasmid shuffle for complementation of *abd1* Δ . *CEN* vD12 plasmids were cotransformed with *CEN* vD1-C (*CEN*) and $2\mu\text{m}$ vD12 plasmids were cotransformed with $2\mu\text{m}$ vD1-C ($2\mu\text{m}$).

^b +++ indicates colony sizes indistinguishable from those of strains bearing wild-type *ABD1*, ++ indicates slightly reduced colony sizes, + indicates that only pinpoint colonies were formed, and - indicates no growth.

^c WT, wild type.

pensated by simple mass action, whereby increasing the concentration of both subunits by raising gene copy number allows formation of a threshold level of active heterodimer that suffices for yeast viability.

DISCUSSION

Virus-encoded cap methylating enzymes are potential targets for antiviral drugs, because the pharmacological sensitivities of the viral cap methylation reactions can differ from those of the host cell. This notion is supported by the broad-spectrum efficacy of *S*-adenosyl homocysteine (SAH) hydrolase inhibitors against poxviruses and many RNA viruses that encode their own cap-methylating enzymes (2, 10). SAH is a direct product and a competitive inhibitor of cap methylation reactions. Pharmacological inhibition of SAH hydrolase elevates intracellular levels of SAH by preventing its cleavage to homocysteine and adenosine. The inference from the antiviral action of SAH hydrolase inhibitors is that viral cap methyltransferases are more sensitive to SAH than are the numerous cellular enzymes that use AdoMet as a substrate. Blocking a cellular enzyme to interdict viral capping is an indirect approach that can be supplanted by targeting the viral cap methylating enzymes directly. The poxvirus cap guanine-N7 methyltransferase is an attractive drug target, because it is uniquely dependent on a virus-encoded regulatory subunit of which there is no homolog in the host cell. The discovery of new antipoxviral drugs is an immediate concern, given the threat of smallpox as a biological weapon and the inevitability of complications as prophylaxis with live vaccinia virus is resumed.

The yeast-based genetic system we describe here provides a valuable tool for basic and applied studies of the poxvirus cap methyltransferase. We show that vaccinia virus cap methyltransferase can function in lieu of the essential yeast enzyme *Abd1* and that its in vivo activity requires coexpression of the catalytic vD1-C and stimulatory vD12 subunits. Hence the methylation function of the viral enzyme can be studied in vivo

independent of the other roles played by the capping enzyme during poxvirus replication (transcription initiation, transcription termination, and telomere resolution). The availability of isogenic yeast strains containing mammalian versus poxvirus cap methyltransferases provides a means of drug discovery aimed at blocking viral cap formation (30). For example, any compound that is selectively cytotoxic to a *vD1-C/vD12* strain but not to an *HCMI* strain is a strong candidate for a specific inhibitor of poxvirus cap methylation.

The yeast genetic system also provides an effective route for identification of amino acids in either subunit that are important for cap methylation. Four individual side chains in the catalytic subunit have been shown to be critical for activity in vivo and in vitro: Asp604, Asp620, Arg632, and Tyr683. All four of these side chains are conserved and essential in cellular cap methyltransferases. Molecular modeling based on the crystal structures of other methyltransferases suggests that the two essential aspartates can contact AdoMet and the essential arginine can interact with the triphosphate bridge of the GpppN cap (4). In the absence of a real atomic structure for any cap guanine-N7 methyltransferase, further genetic and biochemical dissection of structure-activity relationships in *vD1-C* should continue to reveal similarities and distinctions between the catalytic components of the cellular and poxvirus enzymes.

The utility of the yeast-based genetic system extends to the stimulatory *vD12* subunit of the poxvirus capping enzyme. The architecture of the *vD12* side of the subunit interface and the mechanism by which *vD12* binding activates the catalytic subunit are largely uncharted. Substrate-protein cross-linking studies indicate that activation does not entail *D12* stimulation of the binding of AdoMet or the RNA acceptor to the active site on the *vD1-C* subunit (17, 29), suggesting an allosteric effect at the active site perhaps contingent on simultaneous binding of AdoMet and the GpppRNA methyl acceptor (21). Screening in yeast for mutations of *vD12* that display a gene dosage-dependent growth phenotype allows one to identify candidate components of the subunit interface. Moreover, the yeast system offers a potentially powerful means to delineate specific contacts at the subunit interface or the putative allosteric activation site by performing forward genetic screenings for allele-specific suppressors, i.e., selection for single-copy *vD1-C* mutations that suppress single-copy *vD12* mutant phenotypes and vice versa.

Finally, it is noteworthy that the poxvirus cap methyltransferase functions in yeast without attachment to a cellular targeting protein. Capping of pre-mRNA in eukaryotic cells is intimately linked to transcription by RNA Pol II. Cellular capping enzymes are targeted to nascent Pol II transcripts via physical interactions with the phosphorylated carboxyl-terminal domain (CTD) of the largest subunit of Pol II (11, 25). The CTD serves as a scaffold for a multitude of nuclear proteins involved in mRNA maturation (1). Poxviruses replicate entirely in the cytoplasm. Thus, it is sensible that the poxvirus-encoded DNA-dependent RNA polymerase has no counterpart of the CTD (3) and that the poxvirus capping enzyme does not bind to the phosphorylated CTD of nuclear Pol II (25). The earlier finding that the in vivo activity of the vaccinia virus triphosphatase-guanylyltransferase domain in yeast was contingent on its fusion to a cellular CTD-binding protein was taken as evidence in support of the need for a targeting vehicle

to attain genetic complementation of cellular cap-forming enzymes by their viral analogs (18). The present study shows that this does not apply to cap methyltransferase.

An intriguing inference from the complementation of *abd1Δ* by vaccinia virus cap methyltransferase is that there are alternative means to access the 5' ends of pre-mRNAs for guanine N7 methylation that are independent of physical recruitment to the Pol II CTD, to which the vaccinia virus capping enzyme does not bind. The simplest scenario is that the poxvirus capping enzyme is expressed at sufficiently high level under the control of the yeast *TPII* promoter to encounter its RNA substrate in the yeast nucleus without any need for protein-protein interactions with the transcription elongation complex. An alternative scenario, which runs counter to orthodox views of mRNA processing, is that the poxvirus methyltransferase acts on GpppN-capped yeast mRNAs after they have been transported from nucleus to the cytoplasm. Preliminary experiments in which vaccinia virus cap methyltransferase was expressed in yeast as a fusion to green fluorescent protein suggest that the viral enzyme is distributed diffusely throughout the cell. Finer analysis is needed to determine whether the viral cap methyltransferase is acting in the yeast nucleus, the cytoplasm, or both.

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