Mutational Analysis of the Guanylyltransferase Component of Mammalian mRNA Capping Enzyme†

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ABSTRACT: RNA guanylyltransferase is an essential enzyme that catalyzes the second of three steps in the synthesis of the 5′-cap structure of eukaryotic mRNA. Here we conducted a mutational analysis of the guanylyltransferase domain of the mouse capping enzyme Mce1. We introduced 50 different mutations at 22 individual amino acids and assessed their effects on Mce1 function *in vivo* in yeast. We identified 16 amino acids as being essential for Mce1 activity (Arg299, Arg315, Asp343, Glu345, Tyr362, Asp363, Arg380, Asp438, Gly439, Lys458, Lys460, Asp468, Arg530, Asp532, Lys533, and Asn537) and clarified structure−activity relationships by testing the effects of conservative substitutions. The new mutational data for Mce1, together with prior mutational studies of *Saccharomyces cerevisiae* guanylyltransferase and the crystal structures of *Chlorella* virus and *Candida albicans* guanylyltransferases, provide a coherent picture of the functional groups that comprise and stabilize the active site. Our results extend and consolidate the hypothesis of a shared structural basis for catalysis by RNA capping enzymes, DNA ligases, and RNA ligases, which comprise a superfamily of covalent nucleotidyl transferases defined by a constellation of conserved motifs. Analysis of the effects of motif VI mutations on Mce1 guanylyltransferase activity *in vitro* highlights essential roles for Arg530, Asp532, Lys533, and Asn537 in GTP binding and nucleotidyl transfer.

Capping of eukaryotic mRNA occurs by a series of three enzymatic reactions in which the 5′-triphosphate terminus of the pre-mRNA is cleaved to a diphosphate by RNA triphosphatase, capped with GMP by RNA guanylyltransferase, and methylated by RNA (guanine-7) methyltransferase (*1*). The RNA guanylyltransferase component catalyzes a reversible two-step ping-pong reaction (*2*). The first step entails nucleophilic attack of the enzyme at the α-phosphorus of GTP to form a covalent enzyme−(lysyl-N)−GMP intermediate and pyrophosphate (PP_i). In the second step, the β-phosphate of 5′-diphosphate-terminated RNA attacks the enzyme−GMP intermediate to form the GpppRNA cap and expel the lysine nucleophile. Both partial reactions require a divalent cation cofactor.

Cellular and viral RNA guanylyltransferases comprise a branch of the covalent nucleotidyl transferase superfamily, which includes ATP-dependent and NAD^+-dependent DNA ligases, and ATP-dependent RNA ligases (*1*, *3*). Crystal structures of five superfamily members highlight a common fold consisting of an N-terminal nucleotidyl transferase domain and a C-terminal OB fold domain (*4*−*9*). The OB fold consists of a five-stranded antiparallel β-barrel. Within the N-terminal domain is an NMP binding pocket composed of five motifs (I, III, IIIa, IV, and V), which are conserved in order and spacing and define the polynucleotide ligase/mRNA capping enzyme superfamily (*1*) (see Figure 1). Motif I (KxDG) contains the lysine nucleophile to which GMP and AMP become covalently linked in the first step of the capping and ligation reactions, respectively. The OB domains of RNA capping enzymes and ATP-dependent DNA ligases include another conserved structural element, motif VI, which is absent from NAD^+-dependent DNA ligases and ATP-dependent RNA ligases (*10*−*12*).

Crystal structures of *Chlorella* virus guanylyltransferase in GTP-, lysyl-GMP-, and GpppG-bound states underscored important conformational changes during substrate binding and reaction chemistry, especially the opening and closing of the cleft between the nucleotidyl transferase and OB domains (*5*, *6*). The open configuration is one in which the OB domain retroflexes away from the NT domain to widen the cleft, whereas the closed configuration is one in which the OB domain flexes toward the NT domain and narrows the cleft. The capping enzyme must be in the closed conformation to catalyze formation of the enzyme−guanylate (*5*). The effect of domain closure is to bring motif VI of the OB domain into contact with the β- and γ-phosphates of GTP and thereby swing the PP_i leaving group into an apical orientation relative to the attacking lysine nucleophile (see Figure 2). An open guanylyltransferase configuration is presumed to be required at three phases of the catalytic cycle: for GTP binding, for binding of ppRNA, and for release of the capped RNA product.

The 330-amino acid *Chlorella* virus enzyme is the smallest guanylyltransferase known and likely represents the minimal catalytic domain (*13*). Cellular guanylyltransferases are larger and structurally more complex. The RNA guanylyltransferases of fungi such as *Saccharomyces cerevisiae* (Ceg1, 459 amino acids), *Schizosaccharomyces pombe* (Pce1, 402 amino acids), and *Candida albicans* (Cgt1, 449 amino acids)

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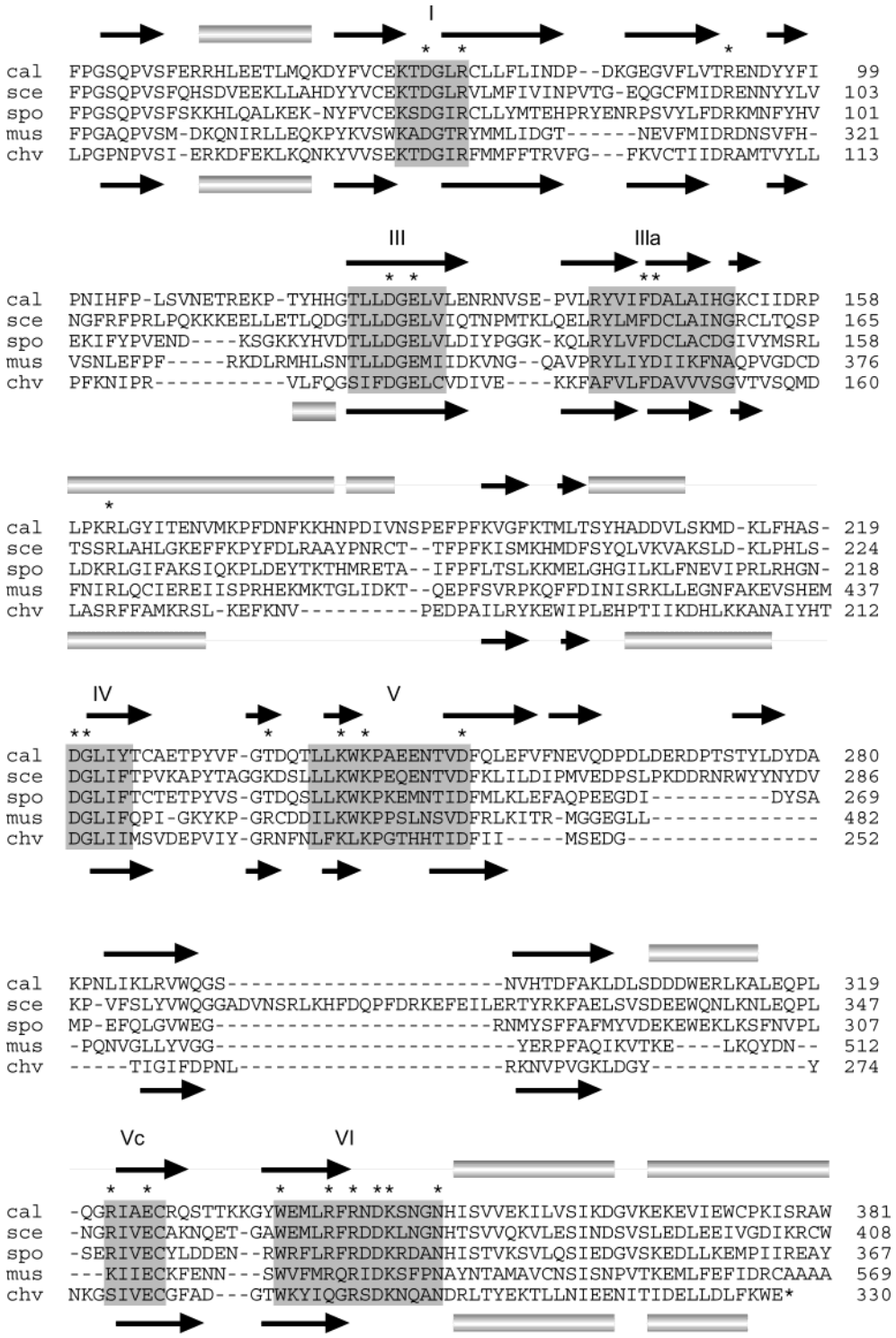

FIGURE 1: Structural conservation in cellular and viral RNA guanylyltransferases. The amino acid sequences of the guanylyltransferases of *C. albicans* (cal), *S. cerevisiae* (sce), *Sc. pombe* (spo), mouse (mus), and *Chlorella* virus PBCV1 (chv) were aligned according to a superposition of the crystal structures of the *Chlorella* virus and *Candida* proteins. Secondary structure elements are shown above and below the aligned polypeptides. Helices are denoted by horizontal bars and β-strands by horizontal arrows. Gaps are indicated by dashes (−). Motifs I, III, IIIa, IV, V, Vc, and VI are highlighted in shaded boxes. Positions in mouse capping enzyme that were targeted for mutational analysis in this study are denoted with asterisks. The conserved proline in motif V demarcates the boundary between the N-terminal nucleotidyl transferase domain and the C-terminal OB domain.

are monofunctional enzymes that are essential for cell growth (*14*−*18*). Their larger size *vis a vis* the minimal *Chlorella* virus enzyme reflects the presence of additional structural elements in their OB domains that mediate interactions with other cellular proteins, including, in the case of budding yeast, the separately encoded RNA triphosphatase component of the capping apparatus (*9*). The mammalian capping enzyme Mce1 is a bifunctional 597-amino acid polypeptide composed of an N-terminal RNA triphosphatase domain (amino acids 1−178) fused to a C-terminal guanylyltransferase domain (amino acids 211−597) (*19*−*23*). All metazoan and plant species for which genome sequences are available encode bifunctional orthologs of the mammalian capping enzyme (*24*−*26*).

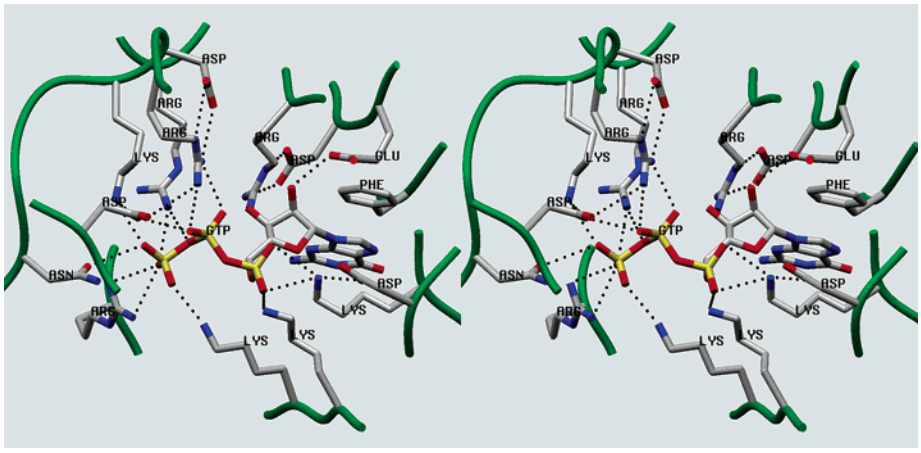

FIGURE 2: Guanylyltransferase active site. The figure shows a stereoview of the GTP-bound active site of *Chlorella* virus guanylyltransferase in the closed conformation (from Protein Data Bank entry 1CKM). Side chains and GTP are colored in CPK mode. The polypeptide backbone is colored green. Electrostatic and hydrogen bond interactions between amino acid side chains and the GTP substrate as well as functionally relevant side chain−side chain interactions are represented by dotted lines. The motif I lysine nucleophile that will become covalently attached to GMP is located to the right of the α-phosphate. The pyrophoshate leaving group is apical to the attacking lysine. Contacts between the motif V lysines and the α- and γ-phosphates are seen at the bottom of the image. There is an extensive array of contacts between the β- and γ-phosphates of GTP and lysine and arginine side chains from motif VI and elsewhere. The image was prepared with SETOR (*49*).

A thorough understanding of the structural basis for the capping reaction and its potential regulation will depend on a comprehensive mutational analysis of exemplary guanylyltransferases, ideally guided by the atomic structure of the enzyme being studied and entailing an assessment of mutational effects on capping activity *in vivo* and *in vitro*. Yet, no one system fits the ideal scenario. The budding yeast *S. cerevisiae* is amenable to facile genetic analysis. Thus, we and others have performed an extensive *in vivo* mutational analysis of the *S. cerevisiae* guanylyltransferase Ceg1, identifying a large number of individual amino acids at which substitutions result in either lethality or a temperature-sensitive growth defect (*10*, *15*, *27*−*33*). Unfortunately, the isolated recombinant yeast guanylyltransferase is less than ideal for biochemical analysis, because of its low activity, its thermolability, and its requirement for interaction with the yeast RNA triphosphatase for optimal function *in vitro* (*33*). The *Chlorella* virus guanylyltransferase, for which a structure is available, is not amenable to genetic analysis, either in the context of the virus (which cannot be manipulated genetically) or by using yeast as a surrogate genetic model; i.e., expression of *Chlorella* virus guanylyltransferase in yeast cannot rescue a *ceg1*Δ mutation (C. K. Ho and S. Shuman, unpublished observations).

The mammalian capping enzyme Mce1 provides an attractive alternative for dissection of the capping mechanism and its regulation. The recombinant triphosphatase and guanylyltransferase domains purified from bacteria are active and thermostable in the absence of other proteins or cofactors (*20*, *33*). The biological function of Mce1 in mammalian mRNA capping is generally accepted, although a formal genetic proof entailing conditional inactivation of Mce1 in mammalian cells is not in hand. In the meantime, the budding yeast *S. cerevisiae* provides a convenient surrogate system for testing the structural requirements for Mce1 function *in vivo*. Expression of the bifunctional mammalian capping enzyme in *S. cerevisiae* complements the growth of yeast strains from which the endogenous triphosphatase (Cet1) or guanylyltransferase (Ceg1) enzymes have been deleted (*19*−*21*, *34*, *35*). Indeed, Mce1 complements growth of the doubly deleted *cet1*Δ*ceg1*Δ strain in which the mammalian protein is the only source of triphosphatase and guanylyltransferase activity (*36*). The atomic structure of the mammalian triphosphatase domain has been determined by X-ray diffraction, and the catalytic mechanism has been elucidated via a combination of biochemical analysis and structure-guided mutagenesis of Mce1 and its baculovirus homologue BVP (*35*, *37*−*39*). In contrast, the atomic structure of the mammalian guanylyltransferase has not yet been determined and structure−activity relationships have received less comprehensive attention (*20*, *21*, *37*).

Here we initiated a systematic molecular genetic analysis of the mammalian guanylyltransferase. We introduced 50 different mutations at 22 individual amino acids and assessed their effects on Mce1 function *in vivo*. The positions chosen for mutagenesis are conserved in the capping enzymes among metazoan, fungal, and *Chlorella* virus capping enzymes and are implicated by the available crystal structures in either GTP binding, phosphoryl transfer chemistry, or stabilization of the active site architecture. We identified a total of 16 amino acids that are essential for Mce1 activity *in vivo* and then clarified structure−activity relationships by introducing conservative substitutions. The new mutational data for Mce1, together with prior mutational studies of yeast Ceg1 and crystal structures for *Chlorella* virus and *C. albicans* guanylyltransferases, provide a coherent view of the functional groups that comprise and stabilize the active site. To understand the role of amino acids in motif VI of the OB domain, we have also analyzed the effects of alanine and conservative mutations of amino acids in this motif on guanylyltransferase activity *in vitro*.

EXPERIMENTAL PROCEDURES

Yeast Expression Plasmids and Site-Directed Mutagenesis. Silent diagnostic restriction sites and single alanine or conservative amino acid substitutions were introduced into the *MCE1* cDNA by PCR using the two-stage overlap extension method as described previously (*35*). The mutated *MCE1* cDNAs were cloned into the yeast vector pYX-His

(*CEN TRP1*) to place *MCE1* expression under the control of a constitutive yeast *TPI1* promoter (*20*). The entire restriction fragment insert was sequenced in every case to confirm the presence of the desired mutation and exclude the acquisition of unwanted coding changes during amplification and cloning.

Test of MCE1 Function by Plasmid Shuffle. S. cerevisiae strain YBS30 (*MAT*a *ura3 ade2 trp1 his3 leu2 can1 ceg1::hisG* pGYCE-360) is deleted at the chromosomal *CEG1* locus, but is viable when it maintains an extrachromosomal copy of *CEG1* on a *CEN URA3* plasmid (p360-CEG1). YBS30 was transformed with pYX-Mce1 plasmids bearing wild-type or mutant alleles of *MCE1*. Trp^+ transformants were selected on medium lacking tryptophan. Two individual colonies were patched on Trp^- medium, and cells from each patch were then streaked on medium containing 0.75 mg/mL 5-fluoroorotic acid (5-FOA). The plates were incubated at 18, 25, 30, and 37 °C. Lethal mutations were those that did not form colonies after 7 days at any of the temperatures. Other mutant alleles supported colony formation within 4 days at one or more temperatures. Two individual FOA-resistant colonies from each strain were patched on YPD agar medium and then streaked on YPD agar at 18, 25, 30, and 37 °C. Growth was assessed by colony size and scored as follows: +++ indicates a colony size indistinguishable from that of strains bearing wild-type *MCE1*, ++ indicates a colony size smaller than that of wild-type *MCE1*, and + denotes pinpoint colonies. Temperature-sensitive (*ts*) mutants were those that did not form colonies at 37 °C after 7 days or formed only pinpoint colonies. Cold-sensitive (*cs*) mutants either failed to form colonies at 18 °C after 10 days or formed only pinpoint colonies.

*Recombinant Mammalian Guanylyltransferase. Nde*I−*Xho*I fragments encoding wild-type or mutated versions of the guanylyltransferase domain Mce1(211−597) were inserted into the T7-based expression plasmid pET16b (Novagen). Cultures (200 mL) of *Escherichia coli* BL21-(DE3)/pET-Mce1(211−597) transformants were grown at 37 °C in Luria-Bertani medium containing 0.1 mg/mL ampicillin until A_{600} reached 0.5. The cultures were adjusted to 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), and incubation was continued at 17 °C overnight with constant shaking. Cells were harvested by centrifugation, and the pellets were stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacterial pellets were resuspended in 10 mL of buffer A [50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, and 10% sucrose] and adjusted to 50 μg/mL lysozyme and 0.1% Triton X-100. The lysate was sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 45 min at 18 000 rpm in a Sorvall SS34 rotor. The soluble extracts were applied to 3 mL columns of Ni−NTA agarose (Qiagen). The columns were washed with buffer A containing 0.1% Triton X-100 and then eluted stepwise with buffer B [50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, and 10% glycerol] containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide compositions of the fractions were monitored by SDS−polyacrylamide gel electrophoresis (PAGE). The recombinant Mce1(211−597) proteins were retained on the column and recovered in the 100 and 200 mM imidazole eluates. The two eluate fractions were pooled and dialyzed against buffer C [50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM DTT, 10% glycerol, and 0.05% Triton X-100]. The protein preparations were stored at −80 °C. Protein concentrations were determined using the Bio-Rad dye binding reagent with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Mutagenesis Strategy. The recently determined crystal structure of the *C. albicans* guanylyltransferase Cgt1 (*9*) allowed us to align the amino acid sequences of mammalian, fungal, and viral RNA guanylyltransferases on the basis of the secondary and tertiary structure landmarks that are conserved in the *Candida* and *Chlorella* virus capping enzymes (Figure 1). The structural alignment underscores the homology of the nucleotidyl transferase motifs (which are shaded in Figure 1), while it rectifies earlier ambiguities in the alignments of the intervening segments, particularly within the C-terminal OB fold domain (*33*). We used this alignment as a guidepost for mutational analysis of Mce1. The strategy was to replace individual residues of interest with alanine and then test the *MCE1-Ala* alleles for *in vivo* activity by complementation of *S. cerevisiae ceg1*Δ. The positions at which alanine substitution elicited a growth defect were targeted for further analysis entailing the introduction of conservative side chain substitutions.

We performed an alanine scan of 22 positions of the mammalian guanylyltransferase indicated with asterisks above the aligned sequences in Figure 1. We focused on positions within nucleotidyl transferase motifs I, III, IIIa, IV, V, and VI, as well as other conserved positions that are implicated in either GTP binding or stabilization of the β-strands that comprise the nucleotide binding pocket of the N-terminal domain. We eschewed analysis of the motif I lysine nucleophile, which has already been shown to be essential for Mce1 function *in vivo* in yeast and for Mce1 guanylyltransferase activity *in vitro* (*19*−*21*).

Alanine Scanning Identifies 16 Mce1 Residues that Are Essential for Guanylyltransferase Activity in Vivo. Twenty-two Mce1-Ala mutants were tested by plasmid shuffle for their ability to function *in vivo* in yeast in lieu of the endogenous guanylyltransferase Ceg1. *MCE1-Ala* alleles were cloned into a yeast *CEN TRP1* plasmid to place their expression under the control of a constitutive promoter. The plasmids were then transformed into a *S. cerevisiae ceg1*Δ strain in which the chromosomal *CEG1* gene was deleted. Growth of *ceg1*Δ is contingent on maintenance of a wild-type *CEG1* allele on a *CEN URA3* plasmid (*27*). Therefore, the *ceg1*Δ strain is unable to grow on agar medium containing 5-FOA (5-fluoroorotic acid, a drug which selects against the *URA3* plasmid) unless it is first transformed with a gene encoding a biologically active guanylyltransferase. Expression of wild-type Mce1 in *ceg1*Δ cells permits their growth on 5-FOA (*20*).

Sixteen of the *Mce1-Ala* transformants failed to give rise to FOA-resistant colonies after incubation for 7 days at 18, 25, 30, or 37 °C; thus, the 16 alanine mutations were lethal *in vivo*. Eleven of the essential residues defined by this analysis were located within the nucleotidyl transferase domain, which extends from the N-terminus to the middle of motif V. The essential residues in this domain are Arg299 (motif I), Arg315, Asp343, and Glu345 (motif III), Tyr362 and Asp363 (motif IIIa), Arg380, Asp438, and Gly439 (motif IV), and Lys458 and Lys460 (motif V) (Table 1). Five

Table 1: Effect of Alanine Mutations on Mce1 Activity *in Vivo*

motif	Mce1 mutation	ceg1Δ complementation	homologous Ceg1 mutation	contacts
I	D296A	*cs*	+++	
	R299A	lethal	lethal	ribose O
	R315A	lethal	*ts*	γ-phosphate
III	D343A	lethal	lethal	Arg299 (motif I)
	E345A	lethal	lethal	ribose O
IIIa	Y362A	lethal	lethal	guanine base
	D363A	lethal	lethal	Arg380
	R380A	lethal	nt	Asp363 (motif IIIa)
IV	D438A	lethal	lethal	
	G439A	lethal	lethal	
	R452A	+++	nt	γ-phosphate
V	K458A	lethal	lethal	γ-phosphate
	K460A	lethal	lethal	α-phosphate
	D468A	lethal	lethal	β-phosphate
Vc	K513A	+++	nt	
	E516A	*cs*, *ts*	+++	Arg530 (motif VI)
VI	W524A	+++	lethal	
	R528A	+++	+++	
	R530A	lethal	lethal	β-phosphate
	D532A	lethal	lethal	Arg530 (motif VI)
	K533A	lethal	lethal	γ-phosphate
	N537A	lethal	nt	γ-phosphate

additional essential residues were located within the OB domain, which extends from the middle of motif V to the C-terminus. The essential residues of the OB domain are Asp468 (motif V) and Arg530, Asp532, Lys533, and Asn537 (motif VI) (Table 1).

Six *Mce1-Ala* mutants that supported colony formation during selection on 5-FOA at either 25 or 30 °C were tested for growth on rich medium (YPD agar) at 18, 25, 30, and 37 °C. Two of the mutants displayed conditional phenotypes. D296A cells were cold-sensitive, growing as well as "wild-type" *MCE1* cells at 25−37 °C, but forming only pinpoint colonies at 18 °C (Table 1). E516A cells grew feebly at 25−30 °C and failed to form colonies at either 18 or 37 °C. Four other *MCE1* strains (R452A, K513A, W524A, and R528A) grew at all temperatures, and their colony sizes were similar to that of wild-type *MCE1* cells. Thus, we surmise that Arg452, Lys513, Trp524, and Arg528 are not essential for guanylyltransferase activity.

Comparison of Alanine Scanning Results for Mammalian and Yeast Capping Enzymes. The homologues of 18 of the 22 positions of Mce1 that were mutated in this study had been subjected previously to *in vivo* mutational analysis in the *S. cerevisiae* guanylyltransferase Ceg1 (*10*, *15*, *27*). There was a high degree of concordance of the mutational results for the two capping enzymes (Table 1). Thirteen of the Mce1-Ala mutations that were lethal *in vivo* were also lethal in the context of Ceg1. These residues in Mce1 are Arg299, Asp343, Glu345, Tyr362, Asp363, Asp438, Gly439, Lys458, Lys460, Asp468, Arg530, Asp532, and Lys533. Eight of these are the counterparts of amino acids that make direct contact with the guanosine nucleotide in the crystal structures of the *Chorella* virus or *C. albicans* capping enzymes (Table 1 and Figure 2) (*5*, *9*). The two side chains that were concordantly nonessential in Mce1 and Ceg1 (Asp296 in motif I and Arg528 in motif VI) are ones that make no contact with GTP or other side chains in the crystal structures. (Although the D296A mutation of Mce1 conferred a *cs* phenotype in yeast, we regard Asp296 as nonessential given that D296A cells grew normally at 25−37 °C. The equivalent alanine mutation of the motif I Asp72 in yeast Ceg1 had no effect on cell growth at 25−30 °C; growth of the *CEG1-D72A* strain at low temperatures was not tested.)

We found several instances of discordance between mutational findings for Mce1 and Ceg1, particularly at conserved residues in the OB domain. For example, whereas alanine in lieu of the motif VI tryptophan (Trp524) had no effect on Mce1 activity in yeast, the equivalent W363A mutation in Ceg1 was lethal (*10*). Ceg1 function was restored when the tryptophan was replaced with phenylalanine, suggesting that the aromatic quality of the side chain is critical in Ceg1. In the guanylyltransferase crystal structures, the motif VI tryptophan is located within the hydrophobic core of the OB domain and is in no position to interact with substrate or participate directly in catalysis (*5*, *9*). We infer that the tryptophan is required to stabilize the enzyme fold in Ceg1, but not in Mce1.

Whereas elimination of the Glu516 side chain in Mce1 resulted in a severe growth defect at permissive temperatures (25−30 °C) and failure to grow at higher or lower temperatures, the equivalent substitution in Ceg1 elicited no growth phenotype (*33*). This glutamate is located within motif Vc ([R/K]IVEC), which is conserved among cellular and *Chlorella* virus guanylyltransferases, but absent from poxvirus capping enzymes and DNA ligases (*10*). This motif Vc glutamate does not contact GTP in the *Chlorella* virus capping enzyme; rather, it engages in a hydrogen bond with the arginine side chain of motif VI (the equivalent of the essential residue Arg530 in Mce1) that contacts the β-phosphate of GTP (*5*). We surmise that Glu516 of Mce1 functions indirectly in capping by correctly positioning the essential motif VI arginine. The motif Vc Glu is apparently not important in the context of yeast Ceg1.

The relative importance of the arginine (Arg315 in Mce1) located midway between motifs I and III also differs in Mce1 and Ceg1. This arginine is conserved in cellular and *Chlorella* virus capping enzymes, ATP-dependent DNA ligases, and T4 RNA ligase 2. The arginine makes contact with the γ-phosphate of the NTP substrate and is essential for the activity of DNA ligase and RNA ligase 2 (*5*, *7*, *47*). Here we found that alanine substitution for Mce1 Arg315 was lethal at all growth temperatures. In yeast Ceg1, a triple mutant in which this arginine and the flanking Asp and Glu positions were replaced with alanine grew as well as wild-type yeast at 25 °C, but was inviable at 37 °C (*15*).

For the study presented here, we targeted four residues of Mce1 that had not been mutated previously in Ceg1: Arg380, Arg452, Lys513, and Asn537. Asn537 in motif VI was essential for Mce1 function *in vivo*; the equivalent asparagine of *Chlorella* virus capping enzyme directly coordinates the γ-phosphate of the GTP substrate (*5*, *10*). A remarkable finding was that Arg452 of Mce1 was nonessential for activity *in vivo*, even though the equivalent arginine in *Chlorella* virus guanylyltransferase coordinates the γ-phosphate of GTP. There is apparently a hierarchy of importance among the several side chains that contact the terminal phosphate, and as noted above for Arg315, the effects of mutating one of these side chains may vary depending on

the source of the enzyme. The benign effects of the R452A mutation on Mce1 function are consistent with the lack of side chain conservation at this position, which is occupied by a threonine in the capping enzymes of *C. albicans* and *Sc. pombe* (Figure 1).

Lys513 of Mce1 is located within motif Vc. This position, though typically a basic residue, is not strictly conserved; i.e., it is occupied by serine in the *Chlorella* virus guanylyltransferase (Figure 1). This side chain is not located near the active site of either the *Chlorella* virus or *Candida* guanylyltransferase, in light of which it is not surprising that Lys513 was nonessential for Mce1 activity.

Arg380 was essential for Mce1 function. Arg380 is conserved in other capping enzymes, is located within an α-helix of the nucleotidyl transferase domain, and forms a buried salt bridge with the essential aspartic acid of motif IIIa (Asp363 in Mce1). The Asp−Arg salt bridge is conserved in the crystal structures of *Chlorella* virus and *C. albicans* guanylyltransferases and *Chlorella* virus DNA ligase (*5*, *7*, *9*). We posit that the ion pair stabilizes the active site fold (see below).

Structure−Activity Relationships at Essential Residues of Mce1. We tested the effects of conservative substitutions at the Mce1 positions defined as essential by alanine scanning (excluding Gly439, for which alanine is already construed to represent a conservative change). Arginine was replaced with lysine, lysine with arginine, glutamate with glutamine and aspartate, aspartate with asparagine and glutamate, asparagine with glutamine and aspartate, and tyrosine with phenylalanine and leucine. Also, the two components of the putative Asp363−Arg380 salt bridge were simultaneously changed to alanine. The 28 new Mce1 mutants were tested by plasmid shuffle for *ceg1*Δ complementation; the results are shown in Table 2. Insights into the structural requirements for GTP binding and nucleotidyl transfer emerge when the mutational data are interpreted in light of the atomic contacts seen in the available crystal structures.

The essential motif I Arg (Arg299 in Mce1) donates a hydrogen bond to a ribose oxygen of the nucleotide; the arginine is held in place by a bidentate salt bridge to the essential motif III Asp (Asp343 in Mce1) (see Figure 2). This Arg−Asp ion pair is conserved in the crystal structures of the *Chlorella* virus and *Candida* guanylyltransferases and the *Chlorella* virus and bacteriophage T7 DNA ligases (*4*, *5*, *7*, *9*). The lethality of the conservative R229K and D343N mutations of Mce1 highlights the importance of the ionic interaction and the requirement for a polyvalent guanidinium group in motif I. The viability of the D343E mutant attests that the Mce1 active site can accommodate the longer glutamate side chain. Similar structure−activity relationships at this Arg−Asp pair apply to yeast Ceg1 (Table 2) (*10*).

The essential motif III glutamate (Glu345 in Mce1) contacts a ribose oxygen in the *Chlorella* virus capping enzyme structure (Figure 2). The carboxylate functional group is crucial, insofar as the isosteric E345Q mutation was lethal. E345D cells formed pinpoint colonies on YPD agar at 25−37 °C and failed to grow at 18 °C. Thus, the shortening of the distance from the main chain to the carboxylate was detrimental to Mce1 function.

The essential Arg315 side chain located between motifs I and III is predicted, on the basis of the crystal structure of *Chlorella* virus capping enzyme, to coordinate the γ-phosphate of GTP. Introduction of a lysine in lieu of Arg315 restored wild-type growth at 30 °C, but R315K cells formed only pinpoint colonies at 18 and 37 °C (scored as *cs* and *ts* in Table 2). We surmise that positive charge at this position is essential for guanylyltransferase activity. The counterpart of this arginine is essential for the adenylyltransferase and ligase activities of T4 RNA ligase 2, and as with Mce1, its replacement with lysine results in restoration of function compared to the catalytically defective alanine mutant (*47*).

Table 2: Effect of Conservative Mutations on Mce1 Activity *in Vivo*

Mce1 mutation	*ceg1*Δ complementation	homologous Ceg1 mutation
D296N	+++	viable
D296E	+++	lethal
R299K	lethal	lethal
R315K	*cs*, *ts*	
D343N	lethal	lethal
D343E	+++	viable
E345Q	lethal	lethal
E345D	+ (*cs*)	viable
Y362F	+++	viable
Y362L	+++	lethal
D363N	lethal	*ts*
D363E	+++	viable
R380K	lethal	
R380A/D363A	lethal	
D438N	lethal	lethal
D438E	++ (*cs*, *ts*)	lethal
K458R	lethal	lethal
K460R	lethal	lethal
D468N	lethal	lethal
D468E	*cs*	viable
E516Q	+++	
E516D	+ (*cs*, *ts*)	
R530K	lethal	lethal
D532N	lethal	lethal
D532E	++ (*cs*)	viable
K533R	+++	viable
N537Q	lethal	
N537D	lethal	

The essential Tyr362 residue in motif IIIa of Mce1 is conserved as an aromatic side chain in capping enzymes, ATP-dependent DNA ligases, T4 RNA ligase 2, and most NAD-dependent DNA ligases. The available crystal structures of capping enzymes and ligases show that the motif IIIa aromatic side chain stacks on the purine base (*4*−*9*) (see Figure 2). The finding that replacement of the Mce1 tyrosine with leucine elicited no growth phenotype (in contrast to the lethal Y362A mutation) implies that the aromatic moiety is not strictly required for Mce1 function and can be replaced with a partially isosteric hydrophobic side chain. Note that the equivalent leucine change was lethal in the context of the yeast guanylyltransferase (*10*). Yet, leucine is naturally present at the equivalent motif IIIa position of *Thermus thermophilus* DNA ligase, an NAD^+-dependent enzyme. In the case of *E. coli* DNA ligase, replacing the motif IIIa aromatic residue with alanine did not affect ligase function *in vivo*, although it did reduce ligase affinity for NAD^+ *in vitro* (*40*). Again, the instructive point is that although certain conserved residues of capping enzymes and ligases make similar contacts to the nucleotide substrate, individual enzymes rely to a greater or lesser extent on a given contact for their function.

The Asp363 and Arg380 side chains of Mce1 are predicted, according to the crystal structure of *Chlorella* virus

capping enzyme, to form a salt bridge within the globular core of the nucleotidyl transferase domain. The salt bridge tethers the back end of the motif IIIa β-strand containing the Tyr362 residue that forms a hydrophobic pocket for the guanine base. The replacement of only one member of an ion pair with alanine eventuates in an unopposed charged residue within the protein core, which may by itself destabilize the structure of the mutant enzyme (*41*). Thus, the effects of the single D363A and R380A mutations do not indicate whether the salt bridge is inherently essential. To address this issue, we tested the effects of a double-alanine mutation (R380A/D363A). The rationale was that if an unopposed buried charge was responsible for inactivating Mce1, then the double-Ala mutation should restore activity. This is not what was observed; the R380A/D363A mutation was lethal at all temperatures, just like the single-Ala mutants (Table 2). Conservative changes D363N and R380K were also lethal, indicating that a bidentate ionic interaction between the two residues is critical for Mce1 function. In contrast, the equivalent Asp-to-Asn mutant of yeast Ceg1 was viable at 25 °C, albeit not at 37 °C (Table 2) (*10*, *31*, *32*). The normal growth of the D363E mutant implies that the longer glutamate side chain is easily accommodated within the interior of the Mce1 nucleotidyl transferase domain (Table 2). The equivalent Asp-to-Glu mutant of yeast Ceg1 was also viable.

The motif I aspartate (KxDG) is conserved among cellular and viral guanylyltransferases, ATP-dependent and NAD^+-dependent DNA ligases, and RNA ligases. It is remarkable that the motif I Asp is essential in DNA and RNA ligases (*3*, *40*, *42*−*46*), yet dispensable in yeast and mammalian capping enzymes. The *MCE1-D296A* mutant grew normally at physiological temperatures, but not at 18 °C. We found that conservative mutations D296N and D296E restored normal growth at all temperatures that were tested (Table 2). In contrast, Shibagaki et al. (*29*) reported that the equivalent motif I Asp-to-Glu mutation was lethal in yeast Ceg1, although the asparagine substitution was benign. Here again we see context-dependent mutational effects which show that Ceg1 is more sensitive than Mce1 to steric hindrance by the larger glutamate side chain. The motif I Asp makes no direct contact with substrate or other essential residues in any of the available crystal structures of capping enzymes or ligases. The Asp is not required for the formation of the enzyme−NMP intermediate by ligases or guanylyltransferases. Rather, the Asp plays a critical role at subsequent steps of the polynucleotide ligase pathway that do not apply to the capping reaction. Thus, it is unclear why the motif I Asp is so well-conserved in the guanylyltransferase branch of the superfamily.

Replacing the motif IV Asp438 side chain of Mce1 with asparagine was lethal, implying that the carboxylate is essential for capping activity. Introduction of a glutamate at this position restored viability, although the D438E mutant grew slowly at 25 and 30 °C (scored as ++ in Table 2) and formed only pinpoint colonies at 18 and 37 °C. The equivalent Asp-to-Glu mutation of yeast Ceg1 was lethal, suggesting that different capping enzymes vary in their ability to accommodate the longer spacer between the main chain and the carboxylate of glutamic acid. It is noteworthy that the essential motif IV acidic side chain is naturally a glutamate in poxvirus capping enzymes, ATP-dependent DNA ligases, and RNA ligases (*10*, *46*, *48*).

The motif V lysines contact the phosphates of the nucleotide in the available crystal structures of capping enzymes (Figure 2) and ligases. These contacts explain the requirements for positive charge revealed by the mutational analyses. We found that Lys458 and Lys460 of motif V of Mce1 were strictly essential; i.e., arginine substitution at either position was lethal. The equivalent Lys-to-Arg changes were also lethal in yeast Ceg1 (*10*). The two motif V lysines are conserved in ATP-dependent DNA and RNA ligases. Both of the motif V lysines are strictly essential for the *in vitro* nucleotidyl transferase activity of T4 RNA ligase 2; i.e., arginine mutants are inactive (*47*). On the other hand, the functions of the motif V lysines of *Chlorella* virus DNA ligase can be sustained by arginine (to an extent of 27% of wild-type ligase activity) (*48*).

Replacing the motif V Asp468 side chain of Mce1 with asparagine was lethal, implying that the carboxylate group is essential. Introduction of a glutamate restored wild-type growth at 25 and 30 °C; however, the D468E mutant grew slowly at 37 °C and formed pinpoint colonies at 18 °C. Concordant conservative mutational effects were seen at the equivalent motif V aspartate of yeast Ceg1 (Table 2). The motif V aspartate of *Chlorella* virus capping enzyme interacts with the β-phosphate of GTP, with a lysine side chain in motif VI (equivalent to Lys533 in Mce1) that contacts the γ-phosphate of GTP, and with the arginine of motif VI (corresponding to Arg530 in Mce1), which contacts the β-phosphate of GTP (see Figure 2). The closeness of the motif V Asp carboxylate oxygens to the nonbridging β- and γ-phosphate oxygens in the *Chlorella* virus guanylyltransferase crystal is inconsistent with the expected repulsion between two negatively charged species, which, given that the electrostatically neutralized asparagine substitution was lethal in Mce1 and Ceg1, raises the prospect that the motif V carboxylate engages the PP_i leaving group indirectly via a metal ion coordinated between the β- and γ-phosphates.

Replacement of the Glu516 side chain with aspartate elicited the same severe growth defect as the E516A mutation, i.e., pinpoint colonies at permissive temperatures (25−30 °C) and failure to grow at 18 or 37 °C (Table 2). In contrast, the E516Q mutant grew normally. We surmise that hydrogen bonding potential is the key property of this functional group and that there is a minimum distance requirement from the main chain that is met by Glu or Gln, but not Asp. The conservative mutational effects at the motif Vc Glu are consistent with the suggestion made in the preceding section that this residue may coordinate an essential motif VI arginine via a hydrogen bond.

Motif VI of Mce1 includes four essential amino acids, three of which (Arg530, Lys533, and Asn537) are predicted from the *Chlorella* virus capping enzyme structure to contact the β- and γ-phosphates of GTP (Figure 2). The arginine corresponding to Mce1 Arg530 makes a bidentate hydrogen bond to the β-phosphate. A requirement for this bidentate interaction explains why the Mce1 R530K mutation was lethal (Table 2). The lysine equivalent of Mce1 Lys533 contacts the γ-phosphate of GTP and the essential motif V aspartate. The fact that the K533R mutant displayed wild-type growth at all temperatures implies that positive charge is the key property of this residue. The motif VI Asp side

chain coordinates the motif VI arginine in the crystal structures of the *Chlorella* virus and *C. albicans* capping enzymes (*5*, *9*) (Figure 2). This essential interaction apparently requires a carboxylate functional group, insofar as the D532N mutation of Mce1 was lethal and activity was partially restored by introducing a glutamate in lieu of aspartic acid. The D532E strain grew slowly relative to wild-type MCE1 cells at 30 and 37 °C (scored as ++ in Table 2), formed pinpoint colonies at 25 °C, and failed to grow at 18 °C. The conservative mutational effects at the motif VI Arg, Asp, and Lys positions of Mce1 were concordant with those reported previously for yeast Ceg1 (Table 2).

The essential motif VI asparagine (Asn537 in Mce1) is unique to mRNA capping enzymes; the Asn coordinates the γ-phosphate of GTP in the crystal structure of *Chlorella* virus guanylyltransferase. We found that conservative Mce1 mutations N537Q and N537D were lethal *in vivo* (Table 2). We surmise that an amide group is critical and that there is a tight steric constraint on the distance from the main chain to the amide that precludes accommodation of the longer glutamine side chain.

Effects of Motif VI Mutations on Mce1 Activity in Vitro. Ten of the motif VI mutations were introduced into a plasmid vector designed for T7 RNA polymerase-driven expression of the Mce1 guanylyltransferase domain in *E. coli* (*20*). Wild-type and mutant His_{10}-Mce1(211−597) proteins were purified from soluble bacterial extracts by Ni−agarose affinity chromatography. The 46 kDa guanylyltransferase polypeptide was the predominant species detected by SDS−PAGE, and the extents of purification were comparable for mutant and wild-type Mce1(211−597) (Figure 3A).

Guanylyltransferase activity was assayed by label transfer from [α-^{32}P]GTP to the Mce1(211−597) polypeptide to form a covalent enzyme−guanylate complex (EpG). Our previous studies showed that (i) the extent of EpG formation by wild-type Mce1(211−297) was saturated at ≥10 μM GTP and (ii) the transguanylylation reaction was complete after a 5 min reaction at 37 °C (*20*). Here we measured the extent of EpG formation by wild-type and mutant guanylyltransferases as a function of GTP concentration in the range of 5−77 μM (Figure 3B). We estimated that 25−30% of the input wild-type enzyme molecules became labeled with [^{32}P]GMP at saturating levels of GTP. The R530A, D532A, K533A, and N537A mutants were effectively inert over the same range of GTP concentrations; i.e., they formed 0.2−0.8% of the wild-type level of EpG. We infer that the lethality of the motif VI Mce1 mutations *in vivo* can be attributed to their near-total loss of guanylyltransferase catalytic activity.

The effects of conservative mutations on guanylyltransferase activity *in vitro* were mechanistically instructive. The R530K protein was as defective as R530A in EpG formation, consistent with the lethal effects of the lysine substitution on Mce1 function *in vivo*. Apparently, the bidentate interaction of the motif VI arginine with the β-phosphate is critical for GTP binding and/or reaction chemistry. Replacing Lys533 with arginine restored guanylyltransferase activity to wild-type levels, consistent with the recovery of full *in vivo* activity for the *MCE1-K533R* allele in yeast. Thus, a positive charge suffices for the interaction of this motif VI side chain with the γ-phosphate.

Whereas the N537D mutant was just as defective in transguanylylation as N537A, activity was revived to a modest extent by the N537Q substitution. The GTP titration profile of N537Q was shifted dramatically to the right compared to that of the wild-type enzyme. The extents of EpG formation by N537Q at 5, 10, and 77 μM GTP were 1.8, 2.8, and 10% of the wild-type levels, respectively. Although we did not test higher GTP concentrations, it is clear from these results that the Asn-to-Gln change resulted in a severely diminished affinity for GTP. The low residual of activity of the N537Q enzyme apparently did not suffice for yeast cell growth, insofar as the N537Q mutant was lethal *in vivo*.

The D532N change restored a low level of guanylyltransferase activity. A significantly greater gain of function was elicited by changing Asp532 to glutamate. For both D532N and D523E, the GTP titration curves were shifted to the right,

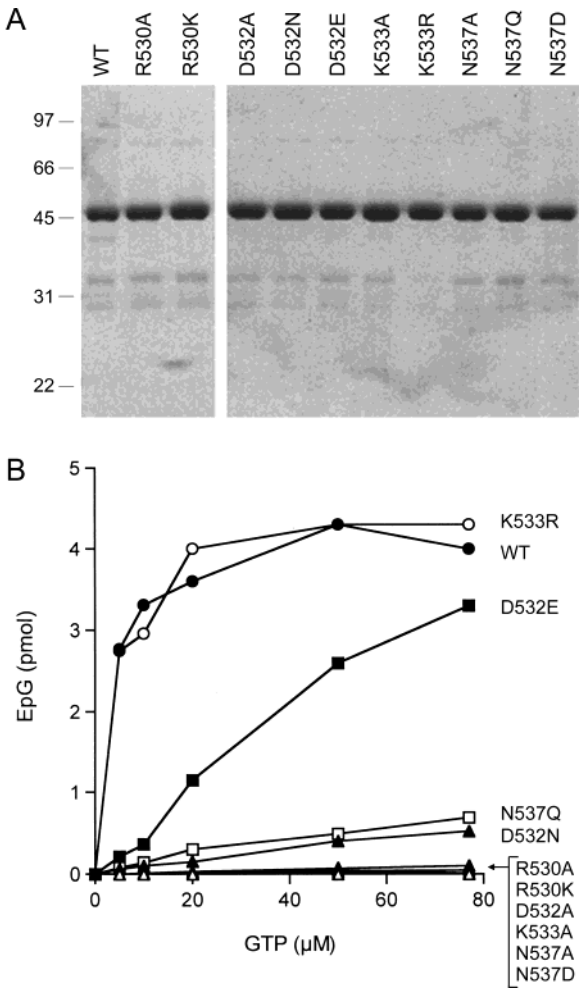

FIGURE 3: Effects of motif VI mutations on guanylyltransferase activity. (A) Aliquots (3 μg) of the Ni−agarose preparations of wild-type Mce1(211−597) and the indicated mutants were analyzed by SDS−PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kilodaltons) of marker polypeptides are indicated at the left. (B) Reaction mixtures (20 μL) containing 50 mM Tris-HCl (pH 8.0), 5 mM DTT, 5 mM $MgCl_2$, 12 pmol (0.6 μM) of recombinant guanylyltransferase, and [α-^{32}P]GTP as specified were incubated for 5 min at 37 °C. Reactions were quenched by adding SDS to a final concentration of 1%. The products were analyzed by SDS−PAGE. Enzyme−[^{32}P]GMP complexes were visualized by autoradiographic exposure of the dried gel and quantitated by scanning the gel with a Fujifilm BAS2500 Bio-Imaging Analyzer. The extent of enzyme−GMP formation is plotted as a function of GTP concentration for each protein as indicated.

indicative of a diminished affinity for GTP. We estimated by interpolation of the curves that the D532E mutation reduced the affinity for GTP by a factor of 20. Note that EpG formation by D532E at higher substrate concentrations was comparable to that of the wild-type capping enzyme. The revived catalytic activity of the D532E mutant was sufficient to sustain a slowed rate of yeast cell growth at physiological temperatures. In contrast, the lower activity of D532N was insufficient to support cell growth (Table 2). These data, together with the findings for N537D, are consistent with a suggestion made previously based on mutational studies of yeast Ceg1 (*20*) that growth of *S. cerevisiae* depends on a threshold level of guanylyltransferase activity.

Comparison with Previous Studies of Mammalian Guanylyltransferase. Other investigators have reported sporadic *in vivo* and *in vitro* mutational data for mammalian guanylyltransferase, some of which agree with our results and some of which do not. Yamada-Okabe et al. (*21*) had shown that mutant alleles R299A (motif I), E354A (motif III), and K458A and K460A (motif V) were unable to complement yeast *ceg1*Δ in a plasmid shuffle assay entailing selection on 5-FOA at 30 °C. Our results establish the unconditional lethality of these alanine mutations at all temperatures from 18 to 37 °C. Wen et al. (*37*) tested the effects of selected motif VI mutations on enzyme−guanylate formation *in vitro*. Their findings that the R530A, R530K, K533A, and N573A substitutions abrogated EpG formation are consistent with the results presented here. However, their report that conservative mutant K533R was catalytically inert is at odds with our data showing that K533R is fully active *in vitro* and *in vivo* and with the data of Wang et al. (*10*) which show that the equivalent Lys-to-Arg mutant of yeast capping enzyme is also active *in vivo*. Wen et al. (*37*) also find that the N537Q mutation completely eliminated the activity of the mammalian guanylyltransferase, whereas we observe that N537Q is partially active *in vitro*. A possible explanation for the disparity is that Wen et al. measured transguanylylation activity in reaction mixtures containing 0.16 μM [α-^{32}P]GTP, which is well below the concentration necessary to attain 50% of the maximal yield of EpG (*20*) and several orders of magnitude lower than the GTP concentrations used in the study presented here. We suspect that mutational effects on EpG formation may have been exaggerated by the use of a single suboptimal substrate concentration, particularly in light of the evidence presented here that conservative motif VI mutations exert strong effects on the GTP concentration dependence of EpG formation.

SUMMARY AND CONCLUSIONS

By testing the effects of 50 mutations at 22 positions in the guanylyltransferase domain of Mce1, we have identified 16 individual amino acids that are essential for capping activity *in vivo* and elucidated structure−activity relationships for each critical functional group. Plausible mechanistic interpretations of the mutational effects on Mce1 activity have been discussed in detail above in light of mutational data that are available for other covalent nucleotidyl transferases and the crystal structures of exemplary guanylyltransferases and DNA ligases. Our results extend and consolidate the hypothesis of a shared structural basis for catalysis among RNA capping enzymes, DNA ligases, and RNA ligase 2. Our study also highlights the critical role played by motif VI in GTP binding and nucleotidyl transfer.

Comparison of the now extensive mutational data for mammalian and budding yeast guanylyltransferases reveals mostly concordant structure−activity relationships at conserved amino acids. However, there are many disparities between the yeast and mammalian results, as discussed in detail above. We conclude that the functions of the conserved amino acids in the RNA capping enzymes, and covalent nucleotidyl transferases generally, may be context-dependent and must therefore be established empirically for any given member of the enzyme superfamily. Efforts are underway to crystallize the mammalian guanylyltransferase domain.

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