Identification of the Vaccinia Virus mRNA Guanyltransferase Active Site Lysine*

(Received for publication, June 3, 1993, and in revised form, August 4, 1993)

Edward G. Niles and Linda Christen
From the Department of Biochemistry, State University of New York, School of Medicine and Biomedical Sciences, Buffalo, New York 14214

The vaccinia virus mRNA capping enzyme is a heterodimeric protein containing subunits of 97 and 33 kDa, the products of genes D1R and D12L, respectively. The enzyme catalyzes the first three reactions in the mRNA cap formation pathway: mRNA triphosphatase, guanyltransferase, and (guanine-7-)methyltransferase. The guanyltransferase reaction proceeds by way of a covalent enzyme GMP (E-GMP) intermediate (Shuman, S. and Hurwitz, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 187-191) in which the GMP is linked to the large subunit through a lysine residue (Toyama, R., Mizumoto, K., Nakahara, Y., Tatsuno, T., and Kaziro, Y. (1983) Eur. J. Biochem. 2, 2195-2201; Roth, M. J., and Hurwitz, J. (1984) J. Biol. Chem. 259, 13488-13494). In order to identify the map position of the guanyltransferase active site lysine residue, high specific activity [32P]E-GMP was prepared. Digestion of the E-GMP with hydroxylamine at pH 9.5 yielded a 31-kDa radioactive fragment derived from amino acids 1-273. Cleavage of E-GMP with cyanogen bromide produced a radioactive peptide of 14 kDa corresponding to the amino acids 242-365. Lysine residues are found at positions 244 and 260.

Rather than relying on nuclear enzymes, vaccinia encodes most, if not all, of the enzymes necessary to express its genes on its replication cycle in the cytoplasm of infected cells (1). The mRNA cap formation pathway: mRNA triphosphatase, guanyltransferase and (guanine-7-)methyltransferase has permitted the identification of lysine 260 as the site of linkage of GMP. This result agrees with the recent report of Cong and Shuman (37), who analyzed the guanyltransferase activity of the dimeric capping enzyme (3, 4). The enzyme catalyzes the first three reactions of the cap formation pathway: mRNA triphosphatase (5, 6), guanyltransferase (2), and (guanine-7-)methyltransferase (2). In addition, the enzyme catalyzes a nucleoside phosphohydrolase activity (5, 7). The 2'-O-methyltransferase activity required to form the cap I structure is catalyzed by a separate viral enzyme (8). Expression in Escherichia coli of the dimeric capping enzyme and active subdomains has permitted a crude localization of the enzyme active sites. The mRNA triphosphatase, guanyltransferase, and ATPase active sites reside in the N-terminal 60 kDa of the D1R subunit (9-11). The (guanine-7-)methyltransferase is present in a dimer consisting of the C-terminal domain of D1R from amino acids 498 to 844 and the D12L subunit (10, 12). A more precise localization of each active site will permit us to determine if each catalytic activity is carried out by an independent domain of the enzyme, which would require the movement of the 5' nucleotide from one site to the next in sequence.

The guanyltransferase reaction proceeds via the formation of a reversible (18) covalent enzyme-E'2-GMP intermediate (13) in which the GMP is bound to a lysine residue in a phosphoamide linkage (14, 15). E-GMP can be generated in vitro and stabilized by the addition of EDTA to prevent the pyrophosphorylation of the lysine GMP bond (13), allowing its isolation. In order to identify the active site lysine, E-GMP was specifically cleaved with sequence-specific chemical reagents cyanogen bromide and hydroxylamine and the products analyzed by gel electrophoresis. Further digestion of cyanogen bromide-cleaved E-GMP with Staphylococcus aureus V8 protease permitted the identification of lysine 260 as the site of linkage to GMP. This result agrees with the recent report of Cong and Shuman (37), who analyzed the guanyltransferase activity of Lys460 substitution mutations. Lys460 lies in the sequence KXDG conserved in other mRNA capping enzymes and also both DNA and RNA ligases (16). Each of these enzymes catalyzes a nucleotidyl transfer reaction through a nucleotidyl enzyme intermediate.

MATERIALS AND METHODS

Capping Enzyme Preparation—Vaccinia virus capping enzyme subunits were expressed simultaneously in E. coli BL21(DE3)pLysS pET3aD1,D12-3, and the enzyme was purified to homogeneity as described (10). Approximately 10 mg of enzyme was prepared from 1200 g of induced cells. The E\text{2-GMP} was estimated to be 8.1 by analysis of the amino acid composition of both subunits by the GCG program PeptideSort. This value is in close agreement with that determined by the method of Bradford and was used in estimating the concentration of enzyme employed in these analyses.

1. M. A. Higman, unpublished observation.
2. The abbreviations used are: E, enzyme; Tricine, N-tris(hydroxymethyl)methylglycine.

*This work was supported by Grant AI-28824 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Formation and Isolation of E-GMP Complex—Enzyme-GMP was formed in a reaction containing 50 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10 mM β-mercaptoethanol, 10⁻⁴ M GTP, and 1 μCi of [³²P]GTP. To isolate E-GMP for structural analyses, a 500-μl reaction was constructed, containing 350 pmol of capping enzyme and 300 μCi of [³²P]GTP at 10 μM GTP, and preincubated in an ice water bath. The reaction was started by the addition of capping enzyme and quenched after 30 s on ice with the addition of EDTA to 10 mM. E-GMP was separated from free GTP by passage over a 1.5 cm x 6 cm Sephadex G-25 column in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS. Fractions were collected, analyzed by gel electrophoresis and stored at −20 °C with little degeneration of the complex.

Identification of the Active Site Lysine—[³²P]E-GMP was pooled and concentrated in a Centricon microconcentrator. Prior to digestion, the protein was precipitated with nine volumes of −20 °C acetone, washed twice with cold acetone, and vacuum-dried. To cleave with cyanogen bromide (20), an aliquot of E-GMP was resuspended in 200 μl of 70% formic acid containing 10 mg/ml cyanogen bromide. After incubation for 20 h at room temperature, the cyanogen bromide and formic acid were blown off in a hood under a stream of nitrogen. The dried peptide was resuspended in H₂O and redried three times. For analysis of the size of the peptide fragments, the sample was resuspended in gel electrophoresis sample buffer and separated by size in a Tricine gel (19). To cleave with hydroxylamine (21), the acetone-precipitated E-GMP was resuspended in 15 μl of H₂O. 2 μl of 100 mM iodoacetamide was added, and the sample was incubated at 37 °C for 15 min. The reaction was quenched with the addition of 0.4 μl of 1 x mercaptoethanol, and the protein was diluted with 120 μl of 2 mM NH₄OH, 6 mM guanidine HCl, 2 mM LiOH, pH 9.5. After 4 h at 45 °C, the protein fragments were precipitated with −20 °C acetone, washed twice with cold acetone, dried, and resuspended in 30 μl of gel electrophoresis sample buffer. Fragments were separated on a 10% Laemmli polyacrylamide gel (17). S. aureus V8 protease digestion (22) was carried out on E-GMP that had been cleaved with cyanogen bromide as described above. Rather than resuspending the cyanogen bromide cleavage products in gel electrophoresis buffer, cleaved samples were resuspended in 20 μl of 100 mM NH₄HCO₃, pH 7.8. 20 μl of H₂O containing 0−10 μg of V8 protease was added, and the samples were digested for 16 h at 37 °C. 15 μl of digested sample was mixed with 5 μl of 4 x sample buffer, and the peptides were separated on a Tricine gel (19).

RESULTS

Formation of E-GMP—The ability to form high specific activity E-GMP in vitro at high enzyme and low GTP concentrations is hampered by the presence of an inherent potent nucleoside phosphohydrolase activity (5, 7), which rapidly diminishes the GTP level in the reaction mixture. As a result of the GTase activity and the pyrophosphatase exchange reaction, the level of E-GMP decreases. In order to obtain the highest level of incorporation of GMP, capping enzyme was labeled for short times at low temperature, and, prior to the isolation of the capping enzyme, the active site lysine had previously been localized to the N-terminal 60 residues (20). There are 13 Met residues in the large D1R subunit (9-11), the 13.5-kDa CNBr D1R fragment, which contains two AsnGly linkages in the 844-amino acid D1R subunit (Fig. 1A). In order to identify the exact location of the active site lysine, [³²P]E-GMP was isolated by column chromatography and subjected to sequence-specific chemical cleavage. Hydroxylamine cleaves proteins between asparagine and glycine residues at alkaline pH (21); there are five AsnGly linkages in the 844-amino acid D1R subunit (Fig. 1A). [³²P]E-GMP was incubated with NH₄OH at pH 9.5 at 45 °C. After 4 h, the protein fragments were precipitated with acetone, resuspended in gel sample buffer, and analyzed by gel electrophoresis (Fig. 1B). NH₄OH digestion releases a single 31-kDa radioactive fragment, which corresponds in size to the predicted product of NH₄OH cleavage derived from amino acids 1−273 of the D1R subunit.

Cyanogen bromide cleaves preferentially after methionine residues (20). There are 13 Met residues in the large D1R subunit (Fig. 1A). [³²P]E-GMP was incubated with 10 mg/ml CNBr in 70% formic acid, at 25 °C. After 20 h, the CNBr and formic acid were blown off with nitrogen and the samples were resuspended in gel electrophoresis buffer. Analyses by SDS-gel electrophoresis reveals a single radioactive peptide fragment of 14 kDa molecular mass (Fig. 1B). Since the guanylytransferase active site lysine had previously been localized to the N-terminal 60 kDa of the D1R subunit (9−11), the 13.5-kDa CNBr D1R fragment could be disregarded and the 14.2-kDa fragment was identified as the site of linkage to GMP. This corresponds to a peptide that maps between amino acids 242 and 365 of the D1R subunit.

CLEAVAGE WITH V8 PROTEASE—The results of chemical cleavage of [³²P]E-GMP map the active site lysine to a segment of D1R between amino acids 242 and 273. Two lysine residues exist in this region of the D1R subunit at amino acids 244 and 260 (Fig. 2). In order to identify the correct site of linkage to GMP, it was necessary to further subdivide the protein. S. aureus V8 protease cleaves preferentially at glutamic acids (22). A glutamic acid residue resides at amino acid 253 so that cleavage of...
Vaccinia Virus mRNA Guanyltransferase Active Site

DISCUSSION

The vaccinia virus mRNA capping enzyme catalyzes the first three reactions of the mRNA cap formation pathway. Fundamental to our understanding of the mechanism of cap formation is both the number and the arrangement of the three active sites. It is of interest to ascertain whether 1) the 5' nucleotide of the nascent mRNA binds to a single complex active site, or 2) there are three independent active sites, in which case the 5' nucleotide of the nascent message is first placed in the mRNA triphosphatase active site followed by movement to the guanyltransferase active site and finally to the (guanine-7-)methyltransferase active site. In the latter model, the nascent mRNA chain could serve as a swinging arm responsible for the sequential movement of the 5' nucleotide between active sites.

The guanine-7'-methyltransferase active site has been localized to a subdomain of the D1R subunit, between amino acids 498 and 844. The catalytic activity exhibited by D1R498-844 is activated 30-fold by the binding of the D12L subunit, but the mechanism of activation is not known. A subdomain of the capping enzyme containing D1498-844 is essential for guanyltransferase activity. In order to determine if the exchange of Lys260 with Asn results in a specific alteration in the guanylytransferase activity, we tested the (guanine-7-)methyltransferase-specific activity equivalent to the intact capping enzyme, demonstrating that it is a truly independent catalytic domain.

The GTP-binding amino acid of the guanylytransferase active site has now been physically identified. The catalytic activity exhibited by D1R488-844 is activated 30-fold by the binding of the D12L subunit, but the mechanism of activation is not known. A subdomain of the capping enzyme containing D1498-844 is essential for guanylytransferase activity. In order to determine if the exchange of Lys260 with Asn results in a specific alteration in the guanylytransferase activity, we tested the (guanine-7-)methyltransferase-specific activity equivalent to the intact capping enzyme, demonstrating that it is a truly independent catalytic domain.

The GTP-binding amino acid of the guanylytransferase active site has now been physically identified as lysine 260. Recently, Cong and Shuman (37) constructed a series of site-specific mutations in the vaccinia virus D1R protein in the region of homology to the RNA and DNA ligase AMP-binding site. They showed that the exchange of Lys260 with Asn or Met results in the total loss of the guanylytransferase activity, demonstrating that Lys260 is essential for guanylytransferase activity. In order to determine if the exchange of Lys260 caused a global change in the capping enzyme rather than a specific alteration in the guanylytransferase activity, we tested the (guanine-7-)methyltransferase activity and demonstrated that the Lys260 mutations possess normal levels of (guanine-7-)methyltransferase. From these results they inferred that Lys260 is the GMP-binding amino acid. Since the guanylytransferase activity present in the purified D1R N-terminal 60 kDa subdomain is apparently fully active, it also functions as a domain independent of the (guanine-7-)methyltransferase.

Although the mRNA triphosphatase and nucleoside phosphohydrolyase active sites have been mapped to the N-terminal 60 kDa of the D1R subunit (9-11), their precise locations remain to be determined. However, in competition studies it has been shown that ATP, which does not serve as a substrate for the guanylytransferase reaction, also does not inhibit the formation

3 L. A. Christen, unpublished observation.

FIG. 3. Digestion of E-GMP with V8 protease. [32P]E-GMP was cleaved with CNBr as described in Fig. 1. The fragments were solubilized in 100 mM NH4CO3 and incubated overnight at 37 °C with different amounts of V8 protease. The digestion products were mixed with gel electrophoresis sample buffer and separated according to size (19). We have observed that the cleavage products of less than 10 kDa in molecular mass do not migrate in accord with the Promega low molecular weight protein markers (see Footnote 1). We have recalibrated the mobility of these marker protein standards by transferring peptide fragments generated by cyanogen bromide cleavage of the C-terminal domain of the D1R subunit to polyvinylidene difluoride membranes and determining their N-terminal amino acid sequence. The molecular sizes indicated at right are determined by this recalibration.
of [32P]E-GMP. This demonstrates that the ATPase and guanylyltransferase active sites are independent.

Based on these results, a model in which vaccinia virus capping enzyme possesses three independent active sites must be favored at this time. One might propose that a region near the 5' end of the nascent mRNA binds to the protein and serves as a hinge around which the mRNA swings from one active site to the next.

A marked homology in active site sequences has been noted among enzymes that catalyze nucleotidyl transfer reactions (16, 37). The sequence KXDG is conserved among all DNA and RNA sequences reported (Fig. 4). In the case of the T4 RNA ligase (24), human DNA ligase I (23), yeast tRNA ligase (32), and now vaccinia virus mRNA capping enzyme, the active site lysine has been physically identified. Site-specific mutation of the lysine residue alters the activity of T4 RNA ligase (38), DNA ligase I (39), and vaccinia virus mRNA capping enzyme (37), further demonstrating its requirement for enzyme activity. Mutations in the conserved glycine residue also result in a complete loss of nucleotide binding activity. The conserved aspartic acid, however, can be exchanged in each enzyme without eliminating the formation of the enzyme nucleotide intermediate. In the case of the T4 RNA ligase (35), the transfer of the AMP residue to the acceptor is inhibited, suggesting a role for this amino acid in the second step of the reaction. In both DNA and RNA ligases, the amino acid adjacent to the lysine is not conserved. In capping enzymes, however, threonine is favored. Mutation of Thr to Val in the vaccinia virus capping enzyme (37) substantially lowers E-GMP formation, suggesting that it may play a role in the first half of the guanylyltransferase activity.

Acknowledgments—We thank Meghan Higman and James Myette for helpful suggestions.

REFERENCES
1. Moss, B. (1990) in Virology (Fields, B. N., Knipe, D. M., Chanock, R. M., Hirsch, M. S., Melnick, J. L., Monath, T. P., and Roizman, B., eds) 2nd Ed., pp. 2079–2111, Raven Press, New York
2. Martin, S. A., Paoletti, E., and Moss, B. (1975) J. Biol. Chem. 250, 9322–9329
3. Morgan, J. R., Cohen, L. K., and Roberts, B. E. (1984) J. Virol. 52, 206–214
4. Niles, E. G., Lee-Chen, G. J., Shuman, S., Moss, B., and Broyles, S. S. (1989) Virology 172, 513–522
5. Tutus, D. J., and Paoletti, E. (1977) J. Biol. Chem. 252, 3092–3098
6. Venkatasean, S., Gershovitz, A., and Moss, B. (1980) J. Biol. Chem. 255, 903–908
7. Shuman, S., Broyles, S. S., and Moss, B. (1987) J. Biol. Chem. 262, 12372–12380
8. Barbosa, E., and Moss, B. (1978) J. Biol. Chem. 253, 7692–7697
9. Shuman, S. (1990) J. Biol. Chem. 265, 11960–11966
10. Higman, M. A., Bourgeois, N., and Niles, E. G. (1992) J. Biol. Chem. 267, 16430–16437
11. Guo, P., and Moss, B. (1990) Proc. Natl. Acad. Sei. U. S. A. 87, 4023–4027
12. Cong, P., and Shuman, S. (1992) J. Biol. Chem. 267, 16424–16429
13. Shuman, S., and Hurwitz, J. (1981) Proc. Natl. Acad. Sei. U. S. A. 78, 187–191
14. Toyama, R., Minamoto, K., Nakahara, Y., Tatsuho, T., and Kastrow, Y. (1983) EMBO J. 2, 2195–2201
15. Roth, M. J., and Hurwitz, J. (1984) J. Biol. Chem. 259, 13488–13494
16. Lindahl, T., and Barnes, D. E. (1992) Annu. Rev. Biochem. 61, 85–121
17. Laemmli, U. K. (1970) Nature 227, 680–686
18. Shuman, S., Surka, M., Fureneaux, H., and Hurwitz, J. (1980) J. Biol. Chem. 255, 11588–11598
19. Schägger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
20. Gross, E. (1976) Methods Enzymol. 11, 238–255
21. Bornstein, P., and Balian, G. (1977) Methods Enzymol. 47, 132–145
22. Drapéau, G. R. (1977) Methods Enzymol. 47, 189–191
23. Tankinsson, A. E., Totty, N. F., Gignburg, M., and Lindahl, T. (1991) Proc. Natl. Acad. Sei. U. S. A. 88, 490–494
24. Thogersen, H. C., Morris, H. R., Rand, K. N., and Gist, M. J. (1985) Eur. J. Biochem. 147, 325–329
25. Armstrong, J., Brown, R. S., and Tsigita, A. (1983) Nucleic Acids Res. 11, 7154–7156
26. Dunn, J. J., and Studier, F. W. (1981) J. Mol. Biol. 148, 303–330
27. Schmitt, M. P., Beck, P. J., Kearney, C. A., Spence, J. L., DiGiovanni, D., Cordrey, P., and Molinex, I. P. (1987) J. Mol. Biol. 185, 479–495
28. Ishino, Y., Shinagawa, H., Makino, K., Tamaura, S., Sekiyama, P., and Nakata, A. (1986) Mol. Gen. Genet. 194, 1–7
29. Baker, D. G., White, J. H. M., and Johnston, L. H. (1985) Nucleic Acids Res. 13, 8323–8337
30. Parker, D. G., and White, L. H. M. (1987) Eur. J. Biochem. 162, 659–667
31. Smith, G. L., Chan, Y. S., and Kerr, S. M. (1989) Nucleic Acids Res. 17, 9051–9062
32. Xu, Q., Teplow, D., Lee, T. D., and Abelson, J. (1990) Biochemistry 21, 6132–6136
33. Niles, E. G., Condit, R. C., Caro, P., Davidson, K., Matusick, L., and Seto, J. (1988) Virology 173, 95–112
34. Upton, C., Stuart, D., and McFadden, G. (1991) Virology 183, 773–777
35. Pena, L., Yanez, R. J., Revilla, Y., Viruela, E., and Salas, M. L. (1993) Virology 193, 319–329
36. Shibagaki, Y., Itoh, N., Yamada, H., Nagata, S., and Minumoto, K. (1992) J. Biol. Chem. 267, 9851–9858
37. Cong, P., and Shuman, S. (1992) J. Biol. Chem. 268, 7256–7260
38. Usery, S., Singh, M., and Gist, M. J. (1987) Biochemistry 26, 1688–1696
39. Kodama, K., Barnes, D. E., and Lindahl, T. (1991) Nucleic Acids Res. 19, 6693–6699