Direct Photolinkage of GTP to the Vaccinia Virus mRNA (Guanine-7-) Methyltransferase GTP Methyl Acceptor Site†

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ABSTRACT: Direct UV photolinkage of [α-^{32}P]GTP to the methyl acceptor site of the vaccinia virus (guanine-7-) methyltransferase was attempted in order to identify the GTP binding region of this enzyme. Low-efficiency photolinkage of GTP to the carboxyl terminal domain of the large subunit, D1R$^{498-844}$, was achieved and shown to be specific by several criteria. The half-saturation value for GTP was determined to be 35 μM which is equivalent to the catalytic K_m for the substrate. GTP photolinkage was shown to be inhibited by GpppA, a substrate for the methyltransferase reaction, better than G^{Me}pppA, the reaction product. The addition of $MgCl_2$, known to prevent GTP from serving as a methyl group acceptor in this reaction, was found to eliminate GTP photolinkage. Finally, AdoHcy, a potent product inhibitor of AdoMet binding, failed to inhibit GTP photolinkage, demonstrating that GTP was not linked to the AdoMet binding site. Chemical cleavage of the GTP-labeled enzyme permitted the identification of multiple radioactive peptides, demonstrating the existence of multiple interaction sites in the carboxyl terminal domain of the D1R subunit. The addition of the small D12L subunit has been shown to activate the (guanine-7-) methyltransferase activity in D1R$^{498-844}$ 30–50-fold. The efficiency of GTP photolinkage to the isolated D1R$^{498-844}$ domain, however, was found to be only marginally effected by the addition of the D12L subunit, demonstrating that this enhancement of mRNA (guanine-7-) methyltransferase activity mediated by D12L was not achieved by altering the strength of GTP binding.

Vaccinia, a member of the poxvirus family, is a double-stranded DNA containing virus that conducts its replication cycle in the cytoplasm of infected cells. In order to carry on this unusual life cycle, the virus has evolved to encode many enzymes usually found in the nucleus required for viral DNA replication and gene expression (Moss, 1990). Vaccinia virus presents an unusual opportunity to apply both biochemical and genetic approaches to investigate the mechanisms of gene transcription and mRNA processing in an eukaryotic cell.

The cap structure at the 5′ end of the viral mRNA is constructed by the following set of reactions (Moss et al., 1976):

$$\text{pppApBpC..} \rightarrow \text{ppApBpC..} + \text{Pi} \quad \text{(I)}$$

$$\text{ppApBpC..} + \text{GTP} \rightarrow \text{GpppApBpC..} + \text{PPi} \quad \text{(II)}$$

$$\text{GpppApBpC..} + \text{AdoMet} \rightarrow \text{G}^{\text{Me}}\text{pppApBpC..} + \text{AdoHcy} \quad \text{(III)}$$

$$\text{G}^{\text{Me}}\text{pppApBpC..} + \text{AdoMet} \rightarrow \text{G}^{\text{Me}}\text{ppp}\text{A}_{\text{Me}}\text{pBpC..} + \text{AdoHcy} \quad \text{(IV)}$$

Reactions I–III are catalyzed by the mRNA triphosphatase (Tutas & Paoletti, 1977; Venkatesan et al., 1980), guanylyltransferase (Martin & Moss, 1975), and (guanine-7-) methyltransferase activities of the viral mRNA capping enzyme (Martin & Moss, 1975), while reaction IV is mediated by a separate viral enzyme, the (nucleoside 2′O-) methyltransferase (Barbosa & Moss, 1978). The mRNA capping enzyme is known to contain two subunits of 97 kDa and 33 kDa (Martin et al., 1975), the products of genes D1R and D12L, respectively (Morgan et al., 1984; Niles et al., 1989). Through the structural analysis of the capping enzyme synthesized in *Escherichia coli* (Shuman, 1989, 1990; Higman et al., 1992, 1994) and enzyme purified from virions (Shuman & Morham, 1990), the mRNA triphosphatase and guanylyltransferase active sites were mapped to the N-terminal 60 kDa of the D1R subunit, while the (guanine-7-) methyltransferase activity was shown to reside in a separate domain containing the carboxyl terminal region of the D1R subunit and the D12L subunit (Cong & Shuman, 1992; Higman et al., 1994).

The (guanine-7-) methyltransferase domain can be synthesized in *E. coli* either as separate subunits or as a dimer containing D1R$^{498-844}$/D12L (Higman et al., 1994). Kinetic analysis of the isolated methyltransferase domain demonstrated that it is kinetically equivalent to the intact capping enzyme (Higman et al., 1994), proving that this active site exists as an independent domain in the mRNA capping enzyme. The AdoMet binding site was localized by UV photolinkage studies to two regions in D1R$^{498-844}$, amino acids 499–579 and 806–844, respectively (Higman & Niles, 1994). The carboxyl terminal domain of the large subunit was shown to possess a low level of (guanine-7-) methyltransferase activity which can be dramatically elevated by the addition of the D12L subunit (Higman et al., 1994). AdoMet photolinkage was shown not to be effected by the presence of D12L, demonstrating that the small subunit does not activate through an alteration of AdoMet binding affinity.

Although 5′ guanylylated RNA is the natural substrate for this enzyme, GTP serves as a competent methyl acceptor (Martin & Moss, 1976). In order to identify the GTP binding site in the (guanine-7-) methyltransferase domain, direct UV

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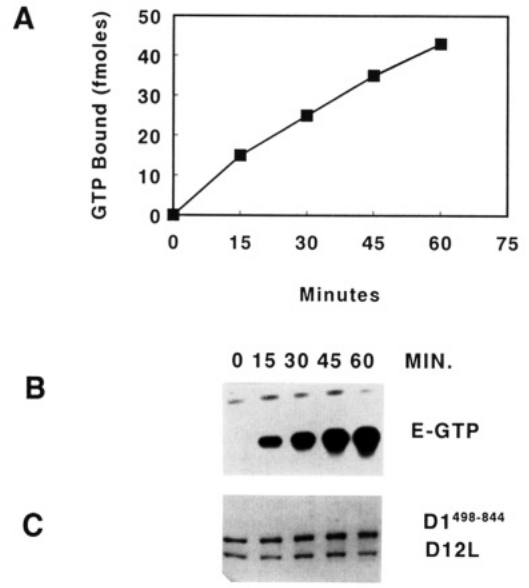

FIGURE 1: Time course of GTP incorporation into the methyltransferase domain. Twenty-five microliter incubations containing 30 pmol of methyltransferase domain, 250 pmol GTP, and 10 μCi of [α-^{32}P]GTP were placed on ice. At different times of irradiation a sample was removed from the UV light and quenched with gel sample buffer. At 60 min, the samples were collected, separated by gel electrophoresis, stained, dried, and autoradiographed. (A) Kinetics of GTP incorporation into D1R$^{498-844}$. (B) An autoradiograph of the gel presented below; E-GTP denotes the position of GTP modified D1R$^{498-844}$. (C) Photograph of the Coomassie Brilliant Blue stained gel with the positions of the methyltransferase domain subunits indicated.

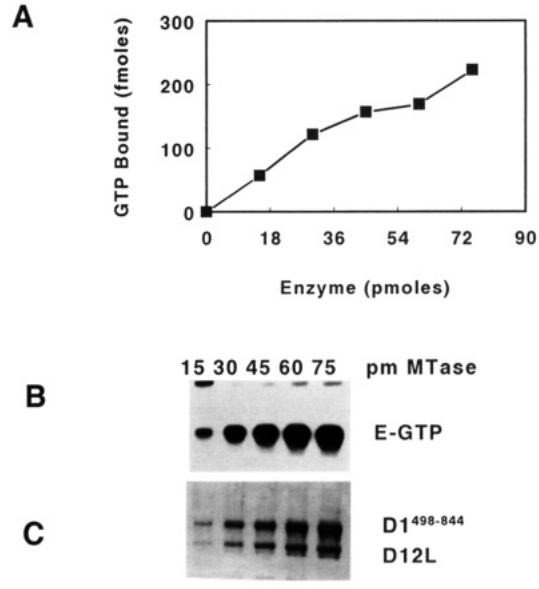

FIGURE 2: Enzyme concentramion dependence of UV photolinkage of GTP to the methyltransferase domain. Twenty-five microliter reactions containing 1250 pmol of GTP, 10 μCi of [α-^{32}P]GTP and from 0 to 75 pmol of methyltransferase domain were irradiated for 60 min in an ice water bath. At the end of the irradiation, the samples were treated as described in Figure 1. (A) A graphical representation of the enzyme concentration dependence of GTP modification of D1R$^{498-844}$. (B) An autoradiograph of the gel electrophoretic separation of the modified enzyme subunits; E-GTP denotes the GTP-D1R$^{498-844}$. (C) Photograph of the Coomassie Brilliant Blue stained gel with the location of the two methyltransferase domain subunits indicated.

photolinkage studies were carried out. In this report we demonstrate that specific low efficiency UV photolinkage of GTP to the D1R$^{498-844}$ domain can be achieved, the primary GTP linkage site is located between amino acids 499 and 620, and linkage to D1R$^{498-844}$ is independent of the small D12L subunit.

EXPERIMENTAL PROCEDURES

Enzyme Purification. The (guanine-7-) methyltransferase domain, D1R$^{498-844}$/D12L, was purified to homogeneity from *E. coli* BL21(DE3) pLysS pET3aD1R$^{498-844}$/D12L, according to Higman et al. (1994). The D12L subunit was isolated from *E. coli* BL21(DE3) pLysS pET 3a D12-8, and D1R$^{498-844}$ was enriched to about 50% purity from *E. coli* BL21(DE3) pLysS pET 8c D1R$^{498-844}$, as described (Higman et al., 1994).

UV Photolinkage Reactions. The standard photolinkage reactions were carried out in a 96-well microtiter dish, floating on an ice water slurry, for 60 min, with a single GE G15T8 15-W germicidal bulb, at a distance of 10 cm. The standard reaction mixture of 25 μL contained 30 pmol of methyltransferase domain, 50 mM Tris HCl, pH 8.0, 10 μM [α-^{32}P]GTP at 1 μCi/25 pmol. In testing the enzyme concentration dependence of photolinkage, the GTP concentration was adjusted to 50 μM. After UV irradiation, 25 μL of 2× Laemmli sample buffer (Laemmli, 1970) were added, the samples were boiled, and 20-μL aliquots were separated by gel electrophoresis. The gels were stained, dried, and autoradiographed. The incorporation of [α-^{32}P]GTP was determined by cutting out the radioactive protein bands and quantifying by liquid scintillation counting. The level of photolinkage was calculated from the measured specific activity of the [α-^{32}P]GTP in the reaction mixture.

To determine the effect of D12L on the efficiency of GTP photolinkage to D1R$^{498-844}$, reactions were set up containing approximately 20 pmol of partially purified D1R$^{498-844}$ and varying amounts of D12L. In order to insure that reactivation of the (guanine-7-) methyltransferase activity had occurred under these conditions, a set of equivalent incubation mixtures were set up lacking [α-^{32}P]GTP, and after incubation on ice for 15 min, an aliquot of each was assayed for (guanine-7-) methyltransferase activity as described (Higman et al., 1994).

Peptide Identification. In order to identify the site of GTP photolinkage a 500-μL reaction mixture was set up. The sample was divided into 10 50-μL aliquots and irradiated as described above. The irradiated samples were pooled and concentrated in an Amicon microcon concentrator, and the labeled protein was separated from the unincorporated ^{32}P-labeled GTP by chromatography on Sephadex G-25, divided into four aliquots, and concentrated by precipitation with 9 volumes of acetone. The protein samples were treated with 10 mg/mL cyanogen bromide or 75% formic acid as described in detail (Higman & Niles, 1994), and the resulting cleavage products were separated by electrophoresis in a Tricine gel (Schagger & von Jagow, 1987). After staining with Coomassie Brilliant Blue, the migration positions of the radioactive peptides were identified by autoradiography. The location of the GTP binding regions in the D1R$^{498-844}$ sequence were deduced from the known sizes of the cleavage products (Higman & Niles, 1994).

RESULTS

UV Photolinkage of GTP to the (Guanine-7-) Methyltransferase. UV irradiation of the methyltransferase domain of the vaccinia mRNA capping enzyme, in the presence of [α-^{32}P]GTP, results in the time-dependent covalent linkage of GTP to the enzyme (Figure 1A). Linkage of GTP occurs almost exclusively to the carboxyl terminal domain of the D1R subunit (Figure 1B,C). In some experiments a minor

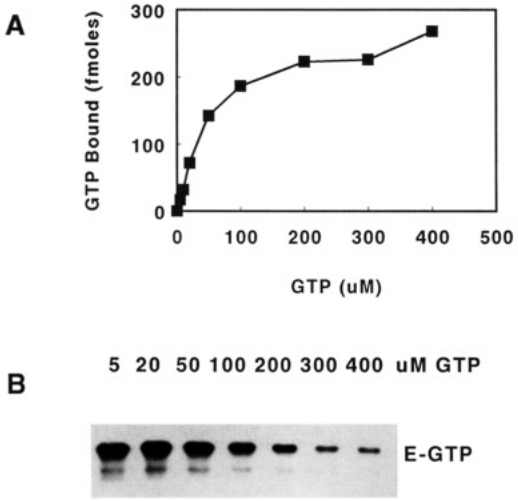

FIGURE 3: GTP concentration dependence of GTP photolinkage to the methyltransferase domain. Reactions containing 30 pmol of methyltransferase domain and 10 μCi of [α-^{32}P]GTP of varying specific activity were irradiated and treated as described in Figure 1. (A) A graphical representation of the GTP concentration dependence of GTP incorporation into the D1R$^{498-844}$ subunit of the methyltransferase domain. (B) An autoradiograph of the gel from which these data were derived; E-GTP denotes the GTP labeled D1R$^{498-844}$.

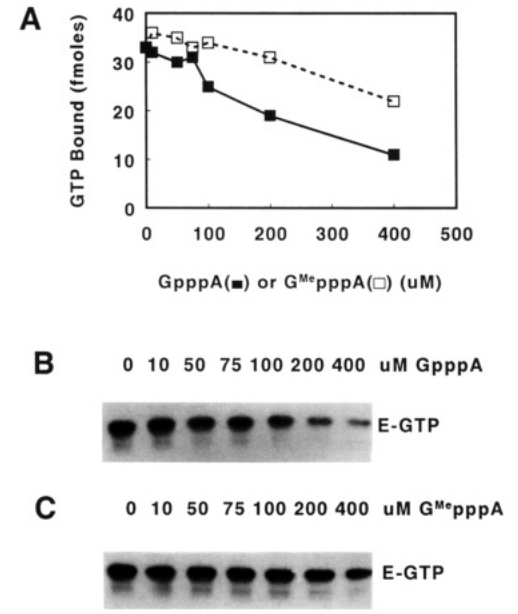

FIGURE 4: Inhibition of GTP photolabeling of the methyltransferase domain by GpppA and G^{Me}pppA. Twenty-five microliter reactions containing 30 pmol of methyltransferase domain, 250 pmol of GTP, 10 μCi of [α-^{32}P]GTP, and varying amounts of GpppA or G^{Me}pppA were irradiated as described in Figure 1. (A) A graphical representation of the inhibitor concentration dependence of inhibition of GTP photolabeling. (B, C) Autoradiographs of the gels from which the GpppA and G^{Me}pppA inhibition data, respectively, were obtained; E-GTP denotes the position of the GTP modified D1R$^{498-844}$.

level of covalent linkage to the D12L subunit can be observed. A low level of UV photocleavage of the protein can be seen in some experiments (Figure 1C). Photolinkage of GTP is linearly dependent on the concentration of enzyme in the reaction mixture (Figure 2A). Again, covalent modification of the D1R$^{498-844}$ subunit is observed exclusively (Figure 2B,C).

Specificity of GTP Photolinkage. A high degree of specificity of GTP photolinkage to the methyltransferase domain is indicated by the preference exhibited for the D1R$^{498-844}$ subunit seen in Figures 1B and 2B. A measurement of the GTP concentration dependence of labeling provides

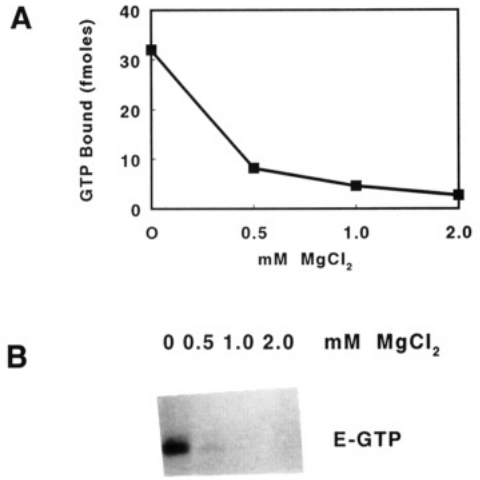

FIGURE 5: Inhibition of GTP photolinkage to the methyltransferase domain by $MgCl_2$. Twenty-five microliter incubations were set up as described in Figure 4, containing varying amounts of $MgCl_2$, and treated as described in Figure 1. (A) A graphical representation of the $MgCl_2$ concentration dependence of inhibition of GTP modification of D1R$^{498-844}$. (B) Autoradiograph demonstrating the incorporation of GTP into D1R$^{498-844}$.

further evidence for specificity. GTP photolinkage is saturatable, exhibiting a $S_{0.5}$ of about 35 μM, Figure 3A,B, which is equivalent to the K_m measured for GTP (Higman et al., 1994). At high GTP concentrations the efficiency of linkage is 0.87%. This low level of photolinkage is due to the low UV photoreactivity observed for guanosine derivatives (Hockensmith et al., 1986, and references therein).

In order to be certain that the observed GTP binding is specific, additional tests were performed. GpppA, a methyl group acceptor substrate of this enzyme (Martin & Moss, 1976), inhibits the efficiency of GTP photolinkage, Figure 4A,B, exhibiting a K_i of about 200 μM, similar to the K_m of 101 μM (Higman et al., 1994), indicating that the GTP is being linked at the active site. G^{Me}pppA, the product of the methyltransferase reaction, is a competitive inhibitor of GTP binding in the methyltransferase assay, exhibiting a K_i of about 900 μM (L. Christen, unpublished). G^{Me}pppA also inhibits GTP photolinkage, Figure 4A,C, with an $S_{0.5}$ of greater than 500 μM, a further indication of photolinkage specificity. ATP is also an effective inhibitor of photolinkage, exhibiting a K_i of about 100 μM, similar to that observed for GTP (data not shown). The methyl acceptor employed in the standard assay is GTP rather than guanylylated RNA. The addition of $MgCl_2$ is known to render MgGTP a poor substrate for methyltransfer (Higman et al., 1994), demonstrating that free GTP is the substrate employed in this reaction. As would be predicted, GTP photolinkage is dramatically inhibited by the addition of low levels of $MgCl_2$, Figure 5A,B, demonstrating that MgGTP is unable to be photolinked to the enzyme, providing further evidence that GTP linkage occurs at the methyltransferase active site. AdoHcy, the product of the reaction, is a potent competitive inhibitor of AdoMet binding, exhibiting a K_i in the 3 μM range (Martin & Moss, 1975; Higman et al., 1994). In order to assess whether GTP may be showing some unexpected affinity for the AdoMet binding region of the methyltransferase active site, AdoHcy was tested as a competitive inhibitor. AdoHcy was found not to be an inhibitor of GTP photolinkage, Figure 6A,B, but rather at low concentrations, to enhance the ability of GTP to be covalently linked to the protein, demonstrating that GTP photolinkage is not occurring at the AdoMet binding locus in the methyltransferase active site.

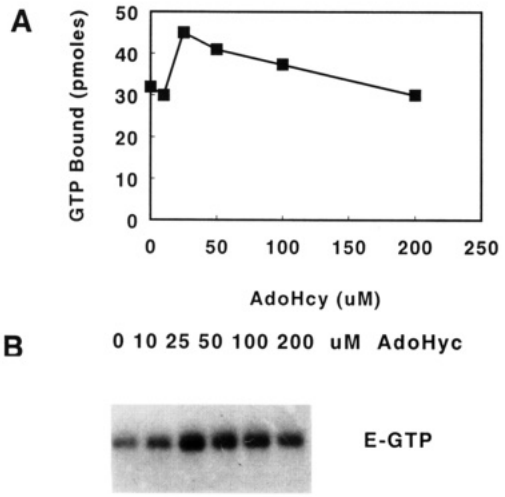

FIGURE 6: Effect of AdoHcy on the efficiency of GTP photolinkage to the methyltransferase domain. Incubations containing varying amounts of AdoHcy were treated as described in Figure 4. (A) Concentration dependence of AdoHcy activation of GTP photolinkage to the D1R$^{498-844}$ subunit. (B) Autoradiograph of gel separation of the GTP modified methyltransferase domain; E-GTP denotes the GTP labeled D1R$^{498-844}$.

Location of the GTP Binding Region. In order to identify the GTP binding region, a large-scale photolinkage reaction was carried out. After removal of the unincorporated radioactivity, the modified protein was subjected to sequence-specific chemical cleavage reactions (Higman & Niles, 1994) (Figure 7A,B). Treatment with acid cleaves at a single site between Asn 620 and Pro 621. Two modified peptides are produced: a major 14.2-kDa photolinked species, derived from amino acids 498–620, and a minor 25.6-kDa carboxyl terminal product. This demonstrates that the GTP binding pocket is derived from multiple regions of the polypeptide chain and that the major site of interaction lies between amino acids 499 and 620. Cleavage with cyanogen bromide at Met residues yields eight products ranging from 0.4 kDa to 13.5 kDa. Two major photolinked peptides are produced: a 9.6-kDa peptide derived from amino acids 499–579 and a 13.5-kDa peptide containing amino acids 580–694. Both modified peptides map in the left end of D1R$^{498-844}$, in agreement with the acid cleavage results. The minor 23-kDa peptide is likely to be a partial cleavage product. A minor photolinkage product of 4.6 kDa derived from the carboxyl terminal 39 amino acids can also be observed.

Effect of D12L on GTP Photolinkage to D1R$^{498-844}$. High levels of (guanine-7-) methyltransferase activity were found to require both the small and large subunits of the mRNA capping enzyme (Cong & Shuman, 1992; Higman et al., 1992). However, a weak (guanine-7-) methyltransferase activity was observed in the isolated D1R$^{498-844}$ which could be activated 30–50-fold by the addition of D12L (Higman et al., 1994). In order to determine whether GTP photolinkage requires the small D12L subunit, the efficiency of GTP linkage to D1R$^{498-844}$ was measured at different levels of D12L. In Figure 8A,B, it can be seen that a modest enhancement of linkage can be observed at 10 μM GTP. Measurement of (guanine-7-) methyltransferase activity demonstrated that full reactivation was achieved (Figure 8C). In order to gain a fuller view of this alteration in GTP binding, the $S_{0.5}$ value for GTP linkage was measured for both D1R$^{498-844}$ and the reconstituted D1R$^{498-844}$/D12L. In Figure 9A,B, it can be observed that in the presence of D12L, GTP photolinkage is slightly more efficient at low GTP concentrations, consistent with Figure 8. However, at high GTP levels, where the (guanine-7-) methyltransferase activity is routinely measured, the D12L subunit has no effect. Therefore, we can conclude that the activation of (guanine-7-) methyltransferase activity by D12L is not mediated through an alteration of the substrate binding strength.

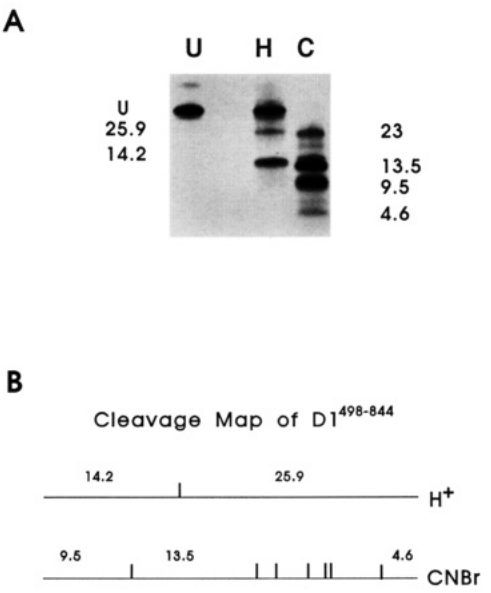

FIGURE 7: Location of the sites of GTP photolinkage. A large-scale photolinkage reaction was carried out, and after reisolation of the modified enzyme, an aliquot was treated with either cyanogen bromide or 75% formic acid. The cleavage products were separated by gel electrophoresis and stained with Coomassie Brilliant Blue, and the migration positions of the modified peptides were determined by autoradiography. The molecular weights of the modified peptides were determined by comparing the autoradiograph with the stained gel. (A) An autoradiograph of the gel separation of the cleavage products. The sizes of the acid cleavage products are listed on the left and the molecular mass of the cyanogen bromide cleavage products are listed on the right. U, uncut; H$^+$, 75% formic acid; C, 10 mg/mL cyanogen bromide. (B) A physical map of the carboxyl terminal domain of the D1R subunit from amino acids 498–844. Acid cleaves between Asn 620 and Pro 621. Cyanogen bromide cleaves at Met residues 498, 579, and 694 yielding the 9.5-kDa and 13.5-kDa peptides, respectively.

DISCUSSION

The vaccinia virus mRNA (guanine-7-) methyltransferase resides in a heterodimer containing the carboxyl terminal domain D1R$^{498-844}$ and the small subunit D12L. *In vivo*, the enzyme employs guanylylated mRNA as a substrate, but *in vitro*, it also can use GTP as a methyl acceptor (Martin & Moss, 1976). The AdoMet binding site was previously localized to two peptides in D1R$^{498-844}$ derived from amino acids 499–579 and 806–844 (Higman & Niles, 1994). In order to identify the GTP methyl acceptor site, further direct photolinkage studies were undertaken.

UV photolinkage of GTP to the (guanine-7-) methyltransferase active site was shown to be dependent upon time of irradiation and the concentration of enzyme in the reaction mixture. Binding was demonstrated to be specific for the active site by several criteria. Photolinkage was shown to be dependent on the GTP concentration, exhibiting an $S_{0.5}$ of 35 μM which is similar to the kinetic K_m for GTP (Higman et al., 1994). Photolinkage is also inhibited by ATP with an $S_{0.5}$ of about 100 μM (data not shown). Although the nucleoside triphosphate binding site shows little ability to discriminate between purine bases, only GTP serves as a methyl acceptor. Photolinkage was also found to be inhibited by GpppA, another

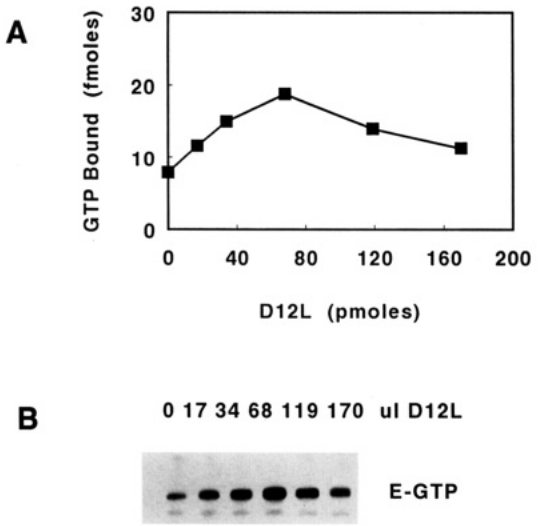

FIGURE 8: Determination of the efficiency of GTP photolinkage to D1R$^{498-844}$ in the presence of varying levels of D12L. Incubations were set up and treated as described in Figure 4, containing partially purified D1R$^{498-844}$ and varying amounts of D12L. (A) A graphical representation of the efficiency of GTP photolinkage to D1R$^{498-844}$ at different levels of D12L. (B) Autoradiograph of the gel electrophoretic separation of the modified methyltransferase subunits; E-GTP denotes the modified D1R$^{498-844}$. (C) Graphical representation of the reactivation of mRNA (guanine-7-) methyltransferase activity by D12L addition.

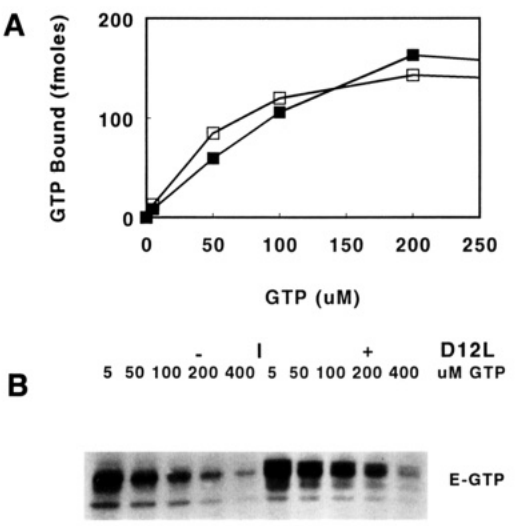

FIGURE 9: GTP concentration dependence of GTP photolinkage to D1R$^{498-844}$ in the absence or presence of the D12L subunit. Incubations containing either D1R$^{498-844}$ alone or in the presence of 68 pmoles of D12L were set up and treated as described in Figure 3. (A) A graphical representation of the GTP concentration dependence of [α-^{32}P]GTP photolinkage to D1R$^{498-844}$ alone (■) or in the presence of 68 pmol of D12L (□). (B) Autoradiograph showing the incorporation of GTP into D1R$^{498-844}$ in the presence or absence of D12L.

methyl accepting substrate, again with a K_i similar to its K_m. G^{Me}pppA, the product of the methyltransferase reaction, was observed to be a relatively poor inhibitor of GTP photolinkage, consistent with its ability to serve as a competitive inhibitor GTP in the methyltransferase reaction. It is known that free GTP is the substrate for this reaction and that Mg^{2+} is a potent inhibitor, presumably through formation of MgGTP (Higman et al., 1994). As a further indication of specificity, Mg^{2+} addition was shown to inhibit GTP photolinkage demonstrating that MgGTP could not participate in the photolinkage reaction. Finally, to test the unlikely possibility that GTP was being linked to the purine binding pocket of AdoMet binding site, AdoHcy, a potent inhibitor of AdoMet binding, was employed as a potential inhibitor of GTP photolinkage. Rather than inhibit, AdoHcy was found to cause a modest enhancement of binding, demonstrating that GTP was not being linked to the AdoMet binding site.

Although photolinkage was shown to be highly specific for the GTP binding site, the efficiency observed at high GTP levels was less than 1%. It is possible that 8-N_3-GTP would be a more photoreactive probe in this analysis. Since the 8-N_3 side group lies adjacent to the methyl acceptor position N_7 on GTP, however, we felt that this compound would be unlikely to serve as a good substrate in the methyltransferase reaction, and in addition, it would be likely to exhibit poor binding.

The low photolinkage efficiency obtained precluded the isolation of modified peptides. However, the regions of close contact to GTP were shown to be limited to the D1R$^{498-844}$ subunit. Furthermore, through analysis of products of sequence specific chemical cleavage of the modified protein, the GTP binding pocket was found to contain portions derived from multiple regions of the protein. On the basis of the products of acid cleavage, 83% of the bound GTP was mapped to the left-hand 14.2 kDa, between amino acids 499 and 620. Cyanogen bromide cleavage yields three radioactive peptides: 94% of the bound GTP is evenly distributed between the 9.5-kDa and 13.5-kDa peptides, between amino acids 499 and 694, in good agreement with the acid cleavage results, while the remaining 6% binds to the 4.6-kDa carboxyl terminal peptide, between amino acids 806 and 844. We can conclude that the major site of GTP photolinkage resides in the left 122 amino acids of the methyltransferase domain. This binding pocket can be further subdivided into at least two regions by cyanogen bromide cleavage. The significance of the minor 4.6-kDa photoproduct remains to be determined. These map positions agree well with the sites identified for AdoMet binding (Higman & Niles, 1994) which were limited to two regions of D1R$^{498-844}$, between amino acids 499–579 and 806–844.

Three amino acid sequence motifs have been associated with GTP binding sites in a variety of GTP hydrolyzing proteins (reviewed in Bourne et al., 1991): GxxxxGK(S/T), NKxD, and DxxG. The only closely related sequence observed in D1R$^{498-844}$ is DYYG, present in amino acids 505–508. GK is found twice at amino acids 670, 671, and 705, 706 without the associated N-terminal G. NKXD is not present. The GTP binding pocket in the (guanosine-7-) methyltransferase does not fit the consensus for GTP hydrolyzing enzymes, but since the natural substrate is guanylylated RNA, this should not be surprising.

A low level of (guanine-7-) methyltransferase activity was found in the isolated D1R$^{498-844}$ subunit (Higman et al., 1994) which could be enhanced 30–50-fold by the addition of the D12L subunit. Two models can account for the activation

caused by D12L binding. In the first, the small subunit might alter the strength of substrate binding so that the catalytic rate could be elevated by D12L addition. In the second model, D12L acts by altering the conformation of the active site such that an amino acid side chain required for catalysis but not substrate binding would be moved into an appropriate position. Higman and Niles (1994) showed previously that D12L binding did not alter the efficiency of AdoMet photolinkage to the $D1R^{498-844}$ subunit. Likewise, in these studies we now demonstrate that the photolinkage of GTP is not substantially affected by the addition of D12L. From these observations we conclude that activation is not achieved through an enhancement of substrate binding to $D1R^{498-844}$ and must deduce that the association of D12L alters the conformation of the active site so that it is more catalytically active.

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