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Identification of the DNA Sequences Encoding the Large Subunit of the mRNA-Capping Enzyme of Vaccinia Virus

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The DNA sequences encoding the large subunit of the mRNA-capping enzyme of vaccinia virus were located on the viral genome. The formation of an enzyme-guanylate covalent intermediate labeled with [α-^{32}P]GTP allowed the identification of the large subunit of the capping enzyme and was used to monitor the appearance of the enzyme during the infectious cycle. This assay confirmed that after vaccinia infection, a novel 84,000-molecular-weight polypeptide corresponding to the large subunit was rapidly synthesized before viral DNA replication. Hybrid-selected cell-free translation of early viral mRNA established that vaccinia virus encoded a polypeptide identical in molecular weight with the ^{32}P-labeled 84,000-molecular-weight polypeptide found in vaccinia virions. Like the authentic capping enzyme, this virus-encoded cell-free translation product bound specifically to DNA-cellulose. A comparison of the partial proteolytic digestion fragments generated by V8 protease, chymotrypsin, and trypsin demonstrated that the ^{32}P-labeled large subunit and the [^{35}S]methionine-labeled cell-free translation product were identical. The mRNA encoding the large subunit of the capping enzyme was located 3.1 kilobase pairs to the left of the *Hin*dIII D restriction fragment of the vaccinia genome. Furthermore, the mRNA was determined to be 3.0 kilobases in size, and its 5′ and 3′ termini were precisely located by S1 nuclease analysis.

The production of mature eucaryotic mRNAs requires transcription of the DNA template, specific processing events, and chemical modification of both the 5′ and 3′ termini of the resulting RNA molecule (28, 50). Each of the 5′ termini of most eucaryotic mRNAs consists of a 7-methylguanosine linked to the penultimate nucleoside by a 5′-5′ triphosphate bridge, designated the cap structure (7me-GpppNp), whereas the 3′ ends of most mRNAs are modified by the posttranscriptional addition of 150 to 200 adenylate residues (2, 11). Enzymes responsible for these modifications have been purified from both cellular and viral systems and have led to an understanding of the basic molecular mechanisms of these modifications (23, 24, 25, 30, 40, 43, 44, 47). Experiments employing microinjection of mRNA into *Xenopus laevis* oocytes suggest that the cap confers resistance to 5′ exonucleolytic degradation in both the nuclear and cytoplasmic compartments and that the 3′ polyadenylic acid [poly(A)] tract is implicated in mRNA stability (14, 16). In vitro studies have also indicated that the cap structure is responsible for ribosome binding and the efficient initiation of translation, presumably mediated by a cellular polypeptide shown to bind to the 5′-terminal cap structure (7, 31, 41). It has been shown that the capping of mRNA chains is an early event in processing and has, in fact, been implicated in the initiation of transcription (13, 18). It remains to be determined whether the capping of nascent RNA chains constitutes an essential function in the initiation of transcription.

Clearly, mutations that alter the pattern of mRNA modification will further elucidate the role of the 5′ cap structure and 3′ poly(A) tract in mRNA biogenesis, translation, and stability. The complexity of the eucaryotic genome precludes the straightforward isolation of mutants in these functions. However, mutants can be easily generated in vaccinia, a DNA virus, which replicates in the cytoplasm and therefore is presumed to encode all of the activities required for mRNA biogenesis and modification (27). Indeed, purified virions contain the full complement of enzymes required to synthesize mature mRNAs of which the DNA-dependent RNA polymerase, the poly(A) polymerase, and the capping enzyme have been purified to homogeneity (10, 24, 25, 29, 30, 32, 44).

As a prelude to the generation of specific mutations, it is essential to identify the DNA sequences encoding these activities. In this report, the gene encoding the large subunit of the capping enzyme was located on the vaccinia genome. The purified capping enzyme was composed of two subunits, 92,500 molecular weight (92.5K) and 26K, and catalyzed the following series of enzymatic reactions resulting in the 5′ cap structure:

(i) $\text{pppNpN-RNA} \xrightarrow{\text{RNA triphosphatase}} \text{ppNpN-RNA} + P_i$

(ii) $\text{GTP} + \text{Enz} \xrightarrow{\text{guanylyltransferase}} \text{EnzpG} + PP_i$

(iii) $\text{EnzpG} + \text{ppNpN-RNA} \xrightarrow{\text{guanylyltransferase}} \text{GpppNpN-RNA} + \text{Enz}$

(iv) $\text{GpppNpN-RNA} + \text{AdoMet} \xrightarrow{\text{guanine-7-methyltransferase}} \text{7meGpppNpN-RNA} + \text{AdoHcy}$

The triphosphate-terminated nascent RNA chain is cleaved by an RNA triphosphatase activity to yield a diphosphate-terminated RNA chain in the first reaction (reaction i)

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(42). Concurrently, the guanylyltransferase reacts with GTP to form an enzyme-guanylate covalent intermediate with the release of pyrophosphate (reaction ii) (39). The GMP of the intermediate is transferred to the 5′ terminus of the diphosphate-terminated RNA to form a triphosphate bridge between the 5′ position of the guanosine and the 5′ position of usually an adenosine or guanosine of the nascent RNA chain (reaction iii). In reaction iv, guanine 7-methyltransferase catalyzes the methylation of the N7 position of the terminal guanine residue, using *S*-adenosylmethionine as the methyl donor (22). This cap structure was further modified by the 2′-O-methylation of the penultimate nucleoside by a separate enzyme, nucleoside-2′-methyl transferase (3).

As a result of the formation of an enzyme-guanylate covalent intermediate by the capping enzyme [reaction (ii)], the largest of the two subunits can be specifically labeled in the absence of an RNA acceptor by [α-^{32}P]GTP (39). To locate the viral DNA sequences encoding the large subunit of the capping enzyme, we took advantage of this labeling assay to identify the [^{35}S]methionine-labeled polypeptide synthesized in the mRNA-dependent reticulocyte lysate which corresponds to the large subunit of the capping enzyme complex. The ^{32}P-labeled large subunit found in vaccinia virions and its corresponding [^{35}S]methionine-labeled cell-free product had identical molecular weights on sodium dodecyl sulfate (SDS)-polyacrylamide gels, affinity for DNA-cellulose, and homologous primary structures as determined by partial enzymatic proteolysis. The mRNA encoding this polypeptide was located on the vaccinia genome by hybrid selection, and its 5′ and 3′ termini were precisely defined by the S1 nuclease procedure.

MATERIALS AND METHODS

Cell culture and virus. Vaccinia virus (WR strain) was a gift from Joseph Kates (Scripps Clinic and Research Foundation, La Jolla, Calif.) and was plaque purified twice in our laboratory. All experiments were carried out with mouse L-cells propagated as monolayers in Dulbecco modified Eagle medium supplemented with 5% calf serum. L-cells were infected with vaccinia virus at a multiplicity of infection equal to 30 in Dulbecco modified Eagle medium supplemented with 5% calf serum. Vaccinia virus was purified by sucrose gradient sedimentation (17). Cytoplasmic extracts from monolayers of infected L-cells were prepared by the method of Sambrook and Shatkin (37).

Isolation of RNA from infected cells. Total cytoplasmic RNA from vaccinia-infected L-cells was prepared by using guanidinium thiocyanate as previously described (21). Early vaccinia RNA was prepared from cells infected in the presence of 100 μg of cycloheximide per ml. The cells were pretreated with cycloheximide for 30 min before infection, and the RNA was isolated at 2 h after the addition of virus.

Translation and polypeptide analysis. RNA was translated in the message-dependent reticulocyte system (New England Nuclear Corp., Boston, Mass.) as previously described (34). Polypeptides were fractionated by electrophoresis in 10% polyacrylamide-SDS gels (19). Fluorography was carried out by the method of Bonner and Laskey, and the dried gels were exposed to Kodak XAR X-ray film at −70°C (5).

Hybrid selection of RNA by DNA immobilized on diazobenzyloxymethyl paper. DNA was immobilized on diazobenzyloxymethyl paper by direct spotting, and filters were hybridized with 100 μg of total cytoplasmic RNA in a reaction volume of 100 μl and processed as previously described (26).

Chromatography of in vitro translation products on DNA-cellulose. DNA-cellulose was made by the method of Alberts and Herrick (1), and DNA-cellulose chromatography was performed in the batch technique as described by Cohen et al. (L. K. Cohen, S. H. Speck, B. E. Roberts, and J. L. Strominger, Proc. Natl. Acad. Sci. U.S.A., in press). Either total cellular RNA or hybrid-selected RNA was used to program the synthesis of [^{35}S]methionine-labeled polypeptides in the reticulocyte lysate system. After translation, 20 μl of the lysates was treated with 2 μl of pancreatic ribonuclease (1 mg/ml) for 30 min at 37°C. After digestion, the lysate was diluted with 180 μl of ice-cold buffer containing 20 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 5 mM mercaptoethanol, 10% glycerol, and 50 mM ammonium acetate. To this dilution was added 40 μl of a 50% suspension of DNA-cellulose equilibrated against the same buffer. Polypeptides were adsorbed to the DNA-cellulose for 60 min at 4°C with mixing. The DNA-cellulose was pelleted by centrifugation in a microcentrifuge for 20 s, and the unbound polypeptides were removed. The DNA-cellulose was subsequently washed five times with 0.5 ml of ice-cold binding buffer with 50 mM ammonium acetate. Each wash was vortexed, and after the DNA-cellulose was pelleted by centrifugation, the washes were removed by aspiration. DNA-binding polypeptides were eluted by the addition of 400 μl of the binding buffer with 1 M ammonium acetate for 5 min on ice. The supernatant containing the eluted polypeptides was saved after pelleting the DNA-cellulose, and the ammonium acetate was removed from the supernatants by three lyophilizations. The DNA-binding polypeptides were dissolved in SDS sample buffer, boiled 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

Preparation and labeling of DNA. The recombinant plasmids containing the *Hin*dIII D through O fragments were generously provided by Bernard Moss (National Institutes of Health, Bethesda, Md.) (4). Digestion of DNA was carried out with restriction enzymes purchased from and used by the procedures outlined by New England Biolabs, Beverly, Mass. DNA fragments were fractionated by electrophoresis in 1% (wt/vol) agarose in 36 mM Tris-hydrochloride (pH 7.6)–36 mM NaH_2PO_4–1 mM Na_2 EDTA. DNA fragments were purified from agarose gels by the method of Vogelstein and Gillespie (48). For 5′ end labeling, DNA restriction fragments were treated with bacterial alkaline phosphatase in 50 mM Tris-hydrochloride (pH 8.0) at 60°C for 15 min. The DNA was extracted three times with phenol and was ethanol precipitated. Fragments were labeled by using [β-^{32}P]ATP and polynucleotide kinase as specified by New England Biolabs. For 3′ end labeling, DNA restriction fragments with 5′ protruding termini were reacted with the Klenow fragment of *Escherichia coli* DNA polymerase, [α-^{32}P]dTTP, and [α-^{32}P]dATP in the restriction enzyme digestion buffer (21).

S1 nuclease analysis and Northern filter hybridization. S1 nuclease mapping was done as described by Sharp et al. (38). Either 5′ or 3′ end-labeled DNA (10 to 50 ng) was mixed with 25 μg of total RNA in a buffer containing 80% deionized formamide, 0.4 M NaCl, 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.4), and 1 mM Na_2 EDTA in a 20-μl reaction. The solution was denatured at 65°C for 10 min and then transferred to a 42°C water bath and allowed to hybridize for 3 h. After hybridization, the reactions were diluted with 200 μl of ice-cold S1 buffer containing 280 mM sodium acetate (pH 4.4), 4.5 mM zinc acetate, 5% glycerol, 25 μg of herring sperm double-stranded DNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml, and 200 U of S1 nuclease (Miles Laboratories, Inc., Elkhart, Ind.). After 30

min of digestion at 37°C, 3 volumes of ethanol were added, and the DNA was precipitated at −20°C. Pelleted DNA was washed twice with 70% ethanol, dried under vacuum, and then fractionated on 8 M urea–5% polyacrylamide gels (21).

Northern filter hybridization was performed as previously described (35). Early viral mRNA was fractionated in formaldehyde agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled DNA fragments.

Labeling of the capping enzyme with [α-^{32}P]GTP. The large subunit of the capping enzyme was labeled with [α-^{32}P]GTP by the method of Shuman and Hurwitz (39) with either purified virions or cytoplasmic extracts of infected L-cells. Labeling was performed in a reaction containing 60 mM Tris-hydrochloride (pH 8.1), 10 mM dithiothreitol, 4 mM $MgCl_2$, 0.05% Nonidet P-40, and 5 μM [α-^{32}P]GTP (550 Ci/mmol). Either 25 μg of total protein from a cytoplasmic extract or 0.36 U (optical density at 260 nm) of purified virions was labeled in a 50-μl reaction. After incubation at 37°C for 5 min, the reaction was stopped on ice by the addition of 5 μl of 0.25 M Na_2 EDTA. Purified virus was pelleted by centrifugation for 5 min in a microcentrifuge and washed twice with 1 mM Tris-hydrochloride (pH 9.0)–1 mM Na_2 EDTA. Cytoplasmic extracts were precipitated on ice for 10 min after the addition of 75 μl of 10% trichloroacetic acid. Precipitated proteins were pelleted and washed twice in anhydrous ether. The precipitates were suspended in SDS gel sample buffer and analyzed on a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was dried, and the labeled polypeptides were visualized by autoradiography. Quantitation of the levels of labeled polypeptide in the extracts was determined by excising the appropriate portions of the dried gel and counting the gel pieces in Betafluor scintillation fluid.

Characterization of polypeptides by partial enzymatic proteolysis in the presence of SDS. Partial enzymatic proteolysis of either [^{35}S]methionine-labeled cell-free translation products or the ^{32}P-labeled large subunit of the capping enzyme was carried out by a modification of the method of Cleveland et al. (8). Polypeptides synthesized in vitro were fractionated in SDS-polyacrylamide gels, and the ^{35}S-labeled polypeptides were visualized by autoradiography. The appropriate portions of the polyacrylamide gel were excised and rehydrated in SDS electrophoresis buffer. Polypeptides were eluted from the gel pieces after being placed in 5-ml glass pipettes which had been plugged with 0.5 ml of 1% agarose in SDS buffer. A 2-in. (5.08-cm) piece of dialysis tubing, 6 mm in diameter, was fitted over the tip of the pipette, filled with 1 ml of SDS buffer, and closed with a dialysis clip. After the pipette was placed in an electrophoresis apparatus for cylindrical gels and filled with SDS buffer, carrier bovine serum albumin (25 μg in 15% glycerol) was layered over the gel pieces. Polypeptides were trapped in the dialysis bag after electrophoresis at 120 V for 12 h. The contents of the dialysis bag were carefully removed, and the bag was washed with 0.3 ml of SDS buffer and 0.3 ml of water. The solutions were pooled, and insoluble material was removed by centrifugation for 5 min in a microcentrifuge. The supernatant was made 20% trichloroacetic acid, and the polypeptides were precipitated on ice for 10 min. The precipitated polypeptides were pelleted by centrifugation for 5 min in a microfuge, and the pellet was washed three times with anhydrous ether. The polypeptides were suspended in a small volume of 10 mM Tris–1 mM Na_2 EDTA (pH 7.0).

Partial enzymatic proteolysis of electroeluted [^{35}S]methionine-labeled cell-free translation products or ^{32}P-labeled capping enzyme polypeptides from vaccinia virions was performed in a solution containing 500 μg of bovine serum albumin per ml, 125 mM Tris-hydrochloride (pH 6.8), 0.5% SDS, 1 mM Na_2 EDTA, 5% glycerol, and an optimal concentration of protease. Either ^{35}S-labeled or ^{32}P-labeled polypeptides (ca. 25,000 cpm) were used for each reaction.

Proteolysis was stopped by the addition of SDS sample buffer and boiling for 2 min. The samples were directly loaded onto an SDS-polyacrylamide gel and fractionated by electrophoresis, and the partial digestion products were visualized by fluorography with the aid of an intensifying screen for the ^{32}P-labeled samples.

RESULTS

Induction of a novel mRNA-capping enzyme upon infection of L-cells with vaccinia virus. During the infection of cells in culture with vaccinia virus, it has been demonstrated that a number of enzymatic activities involved in mRNA biogenesis are rapidly induced before DNA replication (6). To confirm that the induction of a novel mRNA-capping enzyme occurs early in the infection of L-cells with vaccinia virus, the specific labeling of the large subunit of the capping enzyme with [α-^{32}P]GTP was measured (39). L-cells infected with vaccinia virus were harvested at various times; cytoplasmic extracts were prepared and incubated with [α-^{32}P]GTP, and the appearance of the enzyme-guanylate intermediate was monitored after fractionation in an SDS-polyacrylamide gel (Fig. 1).

In the cytoplasmic extract from uninfected cells, two major cellular polypeptides were labeled (Fig. 1A, lanes 1 and 2), whereas upon vaccinia infection, a new 84K polypeptide became apparent. Although there was a slight discrepancy in size between the 84K polypeptide and the purified capping enzyme, this 84K polypeptide was identical in size to the [^{32}P]GMP-labeled polypeptide found in vaccinia virus virions (data not shown). Identical levels of the 84K polypeptide were found at 1 h after infection and in cells blocked by the protein synthesis inhibitor cycloheximide (Fig. 1A, lanes 2 and 8). Therefore, at 1 h after infection, the level of the 84K polypeptide represented the enzyme from the virus inoculum, which has been estimated to contain 60 to 80 capping enzyme molecules per virus particle (39).

In agreement with previous reports, the synthesis of the capping enzyme was rapidly induced upon vaccinia infection, and after 5 h, the level of enzyme was 10-fold greater than the initial input (6). Moreover, when the infection was done in the presence of cytosine arabinoside (araC), an inhibitor of viral DNA replication and thus late viral gene expression, the induction of the capping enzyme was unaffected (Fig. 1A, lane 9). Taken together, these data indicated that the large subunit of the capping enzyme was an early gene product and that its mRNA should be found in the population of mRNAs synthesized at the start of the infectious cycle.

Identification of a virus-encoded cell-free translation product corresponding to the large subunit of the capping enzyme. To establish that the large subunit of the capping enzyme was encoded by the viral genome, the [^{35}S]methionine-labeled polypeptides encoded by mRNA that was hybrid selected to vaccinia DNA fragments were compared with the ^{32}P-labeled subunit. The *Hin*dIII D fragment hybrid selected an early mRNA encoding an 84K polypeptide with an identical molecular weight to the ^{32}P-labeled large subunit found in virions (Fig. 2A). These two polypeptides also comigrated after fractionation by two-dimensional gel electrophoresis (data not shown).

In addition to molecular weight, a distinguishing charac-

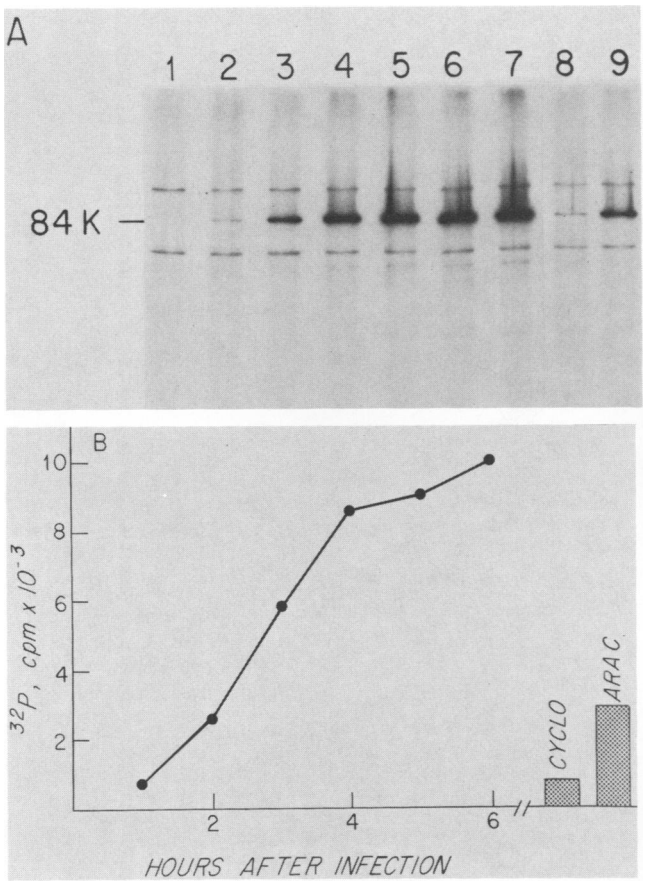

FIG. 1. Induction of the large subunit of the capping enzyme during vaccinia virus infection of L-cells. (A) Autoradiograph of labeled polypeptides after incubation with [α-^{32}P]GTP and fractionation by electrophoresis in an SDS–10% polyacrylamide gel. The dried gel was exposed to film for 20 h. Cytoplasmic extracts were as follows (hours after infection): lane 1, uninfected cells; lane 2, 1.0 h; lane 3, 2.0 h; lane 4, 3.0 h; lane 5, 4.0 h; lane 6, 5.0 h; lane 7, 6.0 h; and lane 8, 2.0 h in the presence of 100 μg of cycloheximide per μl; lane 9, 2.0 h after infection in the presence of 40 μg of araC per ml. (B) Quantitation of enzyme-guanylate intermediate. The polyacrylamide gel slices containing the ^{32}P-labeled enzyme-guanylate complex were excised and counted in scintillation fluid.

teristic of the capping enzyme is its ability to bind to DNA; consequently, DNA-cellulose chromatography is utilized in its purification (14, 25). It has recently been shown that certain cell-free translation products also demonstrate the ability to bind specifically to DNA-cellulose (Cohen et al., in press). As a further indication that the 84K polypeptide corresponded to the large subunit of the capping enzyme, this cell-free product was shown to bind DNA-cellulose (Fig. 2B). Furthermore, two other polypeptides (82K and 26K) encoded by the *Hin*dIII D fragment also demonstrated a strong affinity for DNA-cellulose.

The unambiguous demonstration that the 84K polypeptide was identical to the large subunit of the mRNA-capping enzyme required a comparison of the peptide patterns of the two polypeptides. The separation of the 84K polypeptide and the closely migrating 82K polypeptide encoded by the *Hin*dIII D fragment was achieved by utilizing hybrid selection. The leftward 6.5 kilobases (kb) of the 16-kb *Hin*dIII D fragment were shown to hybrid select the mRNAs encoding both the 84K and 82K polypeptides. This 6.5-kb fragment was subdivided by a *Cla*I site located 3.1 kb from the *Hin*dIII site, and the lefthand *Hin*dIII-*Cla*I fragment exclusively hybrid selected the mRNA encoding the 84K polypeptide (Fig. 2C).

The 84K polypeptide and the large subunit of the capping enzyme have homologous peptide patterns. The large subunit of the capping enzyme and the 84K cell-free translation product were compared by examining the peptide patterns generated by partial enzymatic proteolysis by a modification of the method of Cleveland et al. (8). The large subunit of the capping enzyme packaged in the virion was exclusively labeled with [α-^{32}P]GTP. The [^{35}S]methionine-labeled 84K polypeptide encoded by the mRNA hybrid selected by the *Hin*dIII-*Cla*I fragment was purified by electroelution from an SDS-polyacrylamide gel. An examination of these labeled substrates before enzymatic proteolysis revealed that in addition to the major 84K polypeptide, a number of lower-molecular-weight species were evident (Fig. 3, lanes 1 and 2). The 84K polypeptides repurified from SDS polyacrylamide gels showed the same pattern of lower-molecular-weight species. Therefore, it was concluded that these polypeptides represented spontaneous degradation products, and it is noteworthy that the patterns of degradation of both the [^{35}S]methionine- and ^{32}P-labeled substrates were similar.

After boiling the ^{35}S- or ^{32}P-labeled polypeptides in the presence of SDS, various amounts of one of three proteases, chymotrypsin, trypsin, and V8 protease, were added, and partial proteolysis was conducted for 30 min at 37°C. The limited digestion products were visualized by fluorography after SDS polyacrylamide gel electrophoresis (Fig. 3). With each protease, we obtained similar digestion patterns for the [^{35}S]methionine-labeled 84K polypeptide and the [^{32}P]GMP-labeled large subunit of the capping enzyme. It should be noted that methionine residues would be expected to be located throughout the primary structure of the 84K polypeptide, whereas only one amino acid residue is linked to the GMP (39). This consideration suggests that the patterns of proteolysis would be similar but not necessarily identical and that the greatest correspondence would be found in higher-molecular-weight fragments (Fig. 3). From these data, we conclude that the ^{32}P-labeled large subunit of the capping enzyme and its corresponding [^{35}S]methionine-labeled cell-free translation product have identical primary structures as assayed by partial enzymatic hydrolysis.

Transcriptional map of the gene encoding the large subunit of the capping enzyme. The mRNA encoding the large subunit of the capping enzyme was precisely located on its DNA template by determining the size of the mRNA and the position of its 5′ and 3′ termini. Total early RNA from vaccinia-infected cells was fractionated on a denaturing agarose gel and transferred to nitrocellulose, and the mRNA was visualized by hybridization with the ^{32}P-labeled *Hin*dIII-*Cla*I fragment. A single RNA of 3.0 kb, sufficient in size to encode the 84K polypeptide, was complementary to the *Hin*dIII-*Cla*I fragment (Fig. 4A).

The locations of the 5′ and 3′ termini of this mRNA were ascertained by using a modification of the Berk and Sharp S1 nuclease-mapping procedure (49). The 500-base pair *Bam*HI-*Hin*dIII fragment, 5′ end labeled at the *Bam*HI site, was hybridized to total early RNA and subsequently digested with S1 nuclease. The resultant S1 nuclease-resistant fragment was a measure of the distance from the *Bam*HI site to the 5′ end of the RNA. Two resistant fragments were detected, a major species of 420 nucleotides and a minor one of ca. 430 nucleotides (Fig. 4B). This S1 nuclease analysis

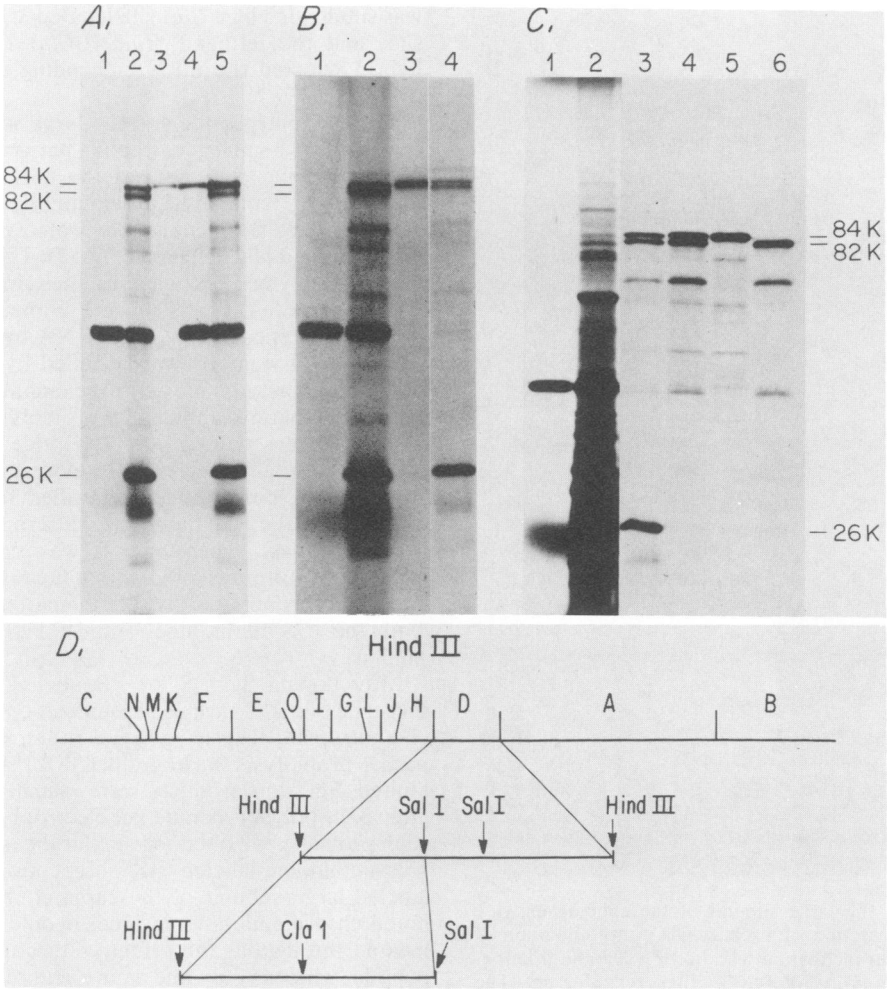

FIG. 2. Comparison of the molecular weights of the ^{32}P-labeled large subunit of the capping enzyme and the [^{35}S]methionine-labeled polypeptides whose synthesis was directed by mRNAs hybrid selected by the *Hin*dIII D fragment. (A) Fluorograph of an SDS–10% polyacrylamide gel of [^{35}S]methionine-labeled polypeptides synthesized in the mRNA-dependent reticulocyte lysate and the ^{32}P-labeled enzyme-guanylate complex from purified virions. The dried gel was fluorographed for 48 h. The cell-free synthesis of [^{35}S]methionine-labeled polypeptides was programmed by mRNA hybrid selected by the *Hin*dIII D fragment. The ^{32}P-labeled large subunit was prepared by incubation of purified vaccinia virions with [α-^{32}P]GTP. Lanes: 1, no mRNA added to the cell-free system; 2, [^{35}S]methionine-labeled polypeptides directed by mRNA hybrid selected by the *Hin*dIII D fragment; 3, ^{32}P-labeled enzyme-guanylate complex from purified virions; 4, mix of lanes 1 and 3; 5, mix of [^{35}S]methionine-labeled polypeptides directed by mRNA hybrid selected by the *Hin*dIII D fragment and ^{32}P-labeled enzyme-guanylate complex from purified virus. (B) Binding of the large subunit to DNA-cellulose. Fluorograph of [^{35}S]methionine-labeled cell-free translation products and ^{32}P-labeled large subunit after fractionation by SDS–10% polyacrylamide gel electrophoresis. RNA hybrid selected to the *Hin*dIII D fragment was used to program the synthesis of [^{35}S]methionine-labeled polypeptides in the mRNA-dependent reticulocyte lysate. After translation, the polypeptides were bound to DNA-cellulose and washed five times with binding buffer, and the DNA-binding polypeptides were eluted with 2 M ammonium acetate in binding buffer. Lanes: 1, no RNA added to the cell-free system; 2, [^{35}S]methionine-labeled polypeptides directed by mRNA hybrid selected by the *Hin*dIII D fragment; 3, ^{32}P-labeled large subunit of the capping enzyme from purified virus; 4, [^{35}S]methionine-labeled DNA-binding polypeptides whose synthesis is directed by mRNA hybrid selected by the *Hin*dIII D fragment. (C) Localization of the mRNA encoding the 84K polypeptide by hybrid selection. Fluorograph of an SDS–9% polyacrylamide gel of [^{35}S]methionine-labeled polypeptides synthesized in the reticulocyte lysate. The dried gel was fluorographed for 15 h for lanes 1 and 2 and 40 min for lanes 3 to 6. The cell-free synthesis of the [^{35}S]methionine-labeled polypeptides was programmed by mRNA hybrid selected by various segments of the *Hin*dIII D fragment. Lanes: 1, no mRNA added to the cell-free system; 2, [^{35}S]methionine-labeled polypeptides directed by total early viral mRNA; 3, mRNA hybrid selected by the *Hin*dIII D fragment; 4, mRNA hybrid selected by the left *Hin*dIII-*Sal*I fragment; 5, mRNA hybrid selected by the *Hin*dIII-*Cla*I fragment; 6, mRNA hybrid selected by the *Cla*I-*Sal*I fragment. (D) Schematic representation of the *Hin*dIII cleavage sites on the vaccinia genome. Expanded view of the *Hin*dIII D fragment and its *Sal*I and *Cla*I sites.

established the direction of transcription and suggested the presence of two closely spaced 5′ termini for the mRNA encoding the large subunit. Heterogeneity of the 5′ termini of a number of vaccinia transcripts has previously been described (45, 46). Consistent with the S1 nuclease data, DNA sequence analysis indicated that an open reading frame for the 84K polypeptide began within 100 nucleotides of the *Hin*dIII site (data not shown).

The 3′ end of the mRNA was located by S1 nuclease analysis by utilizing the 1,100-base pair *Bam*HI-*Cla*I fragment 3′ end labeled at the *Bam*HI site (Fig. 4C). The S1 nuclease-resistant fragment was 800 nucleotides, which indi-

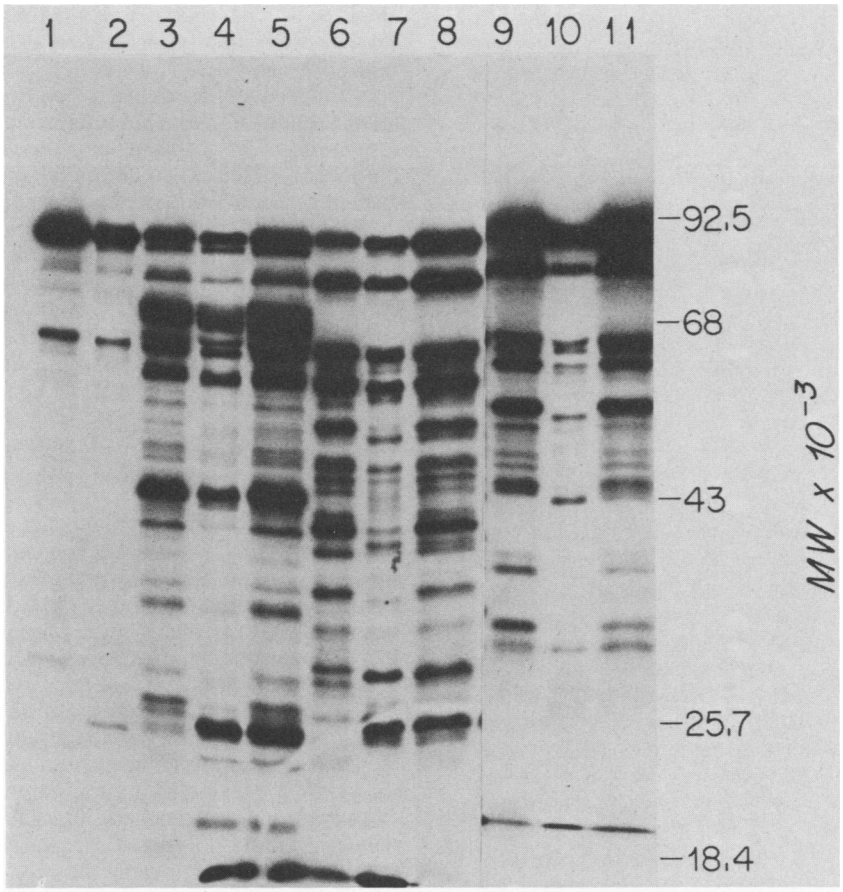

FIG. 3. Comparison of the ^{32}P-labeled large subunit and the [^{35}S]methionine-labeled 84K cell-free translation product by partial enzymatic proteolysis. Fluorograph of the ^{32}P-labeled enzyme-guanylate complex and [^{35}S]methionine-labeled cell-free translation product after fractionation by SDS–10% polyacrylamide gel electrophoresis. RNA hybrid selected to the *Hin*dIII-*Cla*I fragment was used to program the synthesis of [^{35}S]methionine-labeled polypeptides in the mRNA-dependent reticulocyte lysate. After fractionation by SDS-polyacrylamide gel electrophoresis, the 84K [^{35}S]methionine-labeled polypeptide was excised and purified by electroelution. The ^{32}P-labeled enzyme-guanylate complex was prepared by incubation of purified vaccinia virions with [α-^{32}P]GTP. Labeled polypeptides were digested with various proteases for 30 min at 37°C in the presence of SDS and bovine serum albumin. The labeled proteolytic products were fractionated by SDS gel electrophoresis and visualized by fluorography. Lanes: 1, ^{32}P-labeled large subunit with no digestion; 2, [^{35}S]methionine-labeled cell-free product with no digestion; 3, ^{32}P-labeled large subunit digested with 5 μg of V8 protease per ml; 4, [^{35}S]methionine-labeled cell-free product digested with V8 protease; 5, mix of ^{32}P- and ^{35}S-labeled polypeptides digested with V8 protease; 6, ^{32}P-labeled large subunit digested with 5 μg of chymotrypsin per ml; 7, [^{35}S]methionine-labeled polypeptide digested with chymotrypsin; 8, mix of ^{32}P- and ^{35}S-labeled polypeptides digested with chymotrypsin; 9, ^{32}P-labeled large subunit digested with 0.1 μg of trypsin per ml; 10, [^{35}S]methionine-labeled polypeptide digested with trypsin; 11, mix of ^{32}P- and ^{35}S-labeled polypeptides digested with trypsin.

cated that the 3′ end of the mRNA was located this number of nucleotides to the right of the *Bam*HI site. These data established that the 5′ and 3′ ends of the mRNA encoding the large subunit of the capping enzyme were 80 and 300 base pairs from the *Hin*dIII and *Cla*I sites, respectively (Fig. 4D). The S1 nuclease analysis predicted an mRNA of 2.8 kb, which is in close agreement with the 3.0-kb mRNA visualized by Northern filter hybridization. The discrepancy of these two values for the size of the mRNA can be explained by the presence of 150 to 200 adenylate residues on the mRNA as sized by the Northern filter hybridization.

DISCUSSION

The isolation and characterization of over 350 vaccinia virus mutants has defined 32 genetic-complementation groups, and many of these mutations have been located on the viral genome by utilizing marker rescue (9, 12). Although mutants in viral morphogenesis and DNA replication have been defined in this collection, the classification of any mutants as defective in RNA biogenesis has yet to be achieved. Therefore, it is mandatory to develop a biochemical approach for the identification of genes encoding enzymes involved in mRNA biogenesis. This approach to gene localization relies on the identification of the gene product and the direct correspondence between the polypeptide synthesized in vivo and its counterpart produced in a cell-free system. The ideal would be a biochemical activity characteristic of a gene product which can be detected in the corresponding polypeptide synthesized in a cell-free translation system, as demonstrated for the vaccinia thymidine kinase (15). In many cases, however, this approach is undermined by contaminating activities in the cell-free translation systems, the absence of crucial posttranslational modifications, or the inadequate sensitivity of the assay used. In these instances, alternate criteria of identification must be

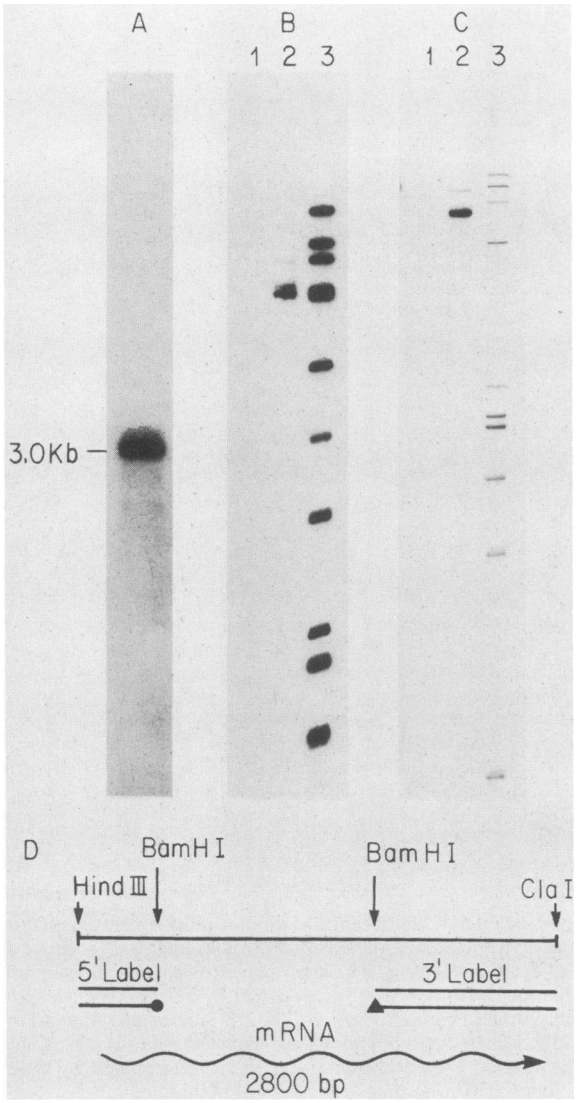

FIG. 4. Determination of the size of the mRNA encoding the large subunit of the capping enzyme and the location of its 5′ and 3′ termini. (A) Autoradiograph of an RNA filter after hybridization with the ^{32}P-labeled *Hin*dIII-*Cla*I fragment. Early RNA (10 μg) was fractionated by electrophoresis in a 1.0% agarose gel containing formaldehyde. After transfer of the RNA onto nitrocellulose, the filter was hybridized with the *Hin*dIII-*Cla*I fragment which had been ^{32}P-labeled by nick translation (36). The filter was exposed to film for 2.0 h. (B) Autoradiograph of 5′ end-labeled S1 nuclease-resistant fragments after fractionation by electrophoresis in an 8 M urea–8% polyacrylamide gel. Plasmid DNA containing the *Hin*dIII-*Cla*I fragment was digested with *Bam*HI and after treatment with calf intestinal phosphatase, was 5′ end labeled by using [β-^{32}P]ATP and polynucleotide kinase. After digestion with *Hin*dIII, the *Hin*dIII-*Bam*HI fragment was purified, hybridized to 25 μg of total early vaccinia RNA, and digested with S1 nuclease. Lanes: 1, no RNA in the hybridization; 2, hybridization with early vaccinia RNA; 3, ^{32}P-labeled *Hin*dI fragments of φX174 DNA, 726 to 713, 553, 500, 427, 417 to 413, 311, 249, 200, 151, 140, and 118 base pairs. (C) Autoradiograph of 3′ end-labeled S1 nuclease-resistant fragments after fractionation by electrophoresis in an 8 M urea–8% polyacrylamide gel. Plasmid DNA containing the *Hin*dIII-*Cla*I fragment was digested with *Bam*HI and 3′ end labeled by using [α-^{32}P]dATP, [α-^{32}P]dTTP, and the Klenow fragment. After digestion with *Cla*I, the *Bam*HI-*Cla*I fragment was purified, hybridized to 25 μg of total early vaccinia RNA, and digested with S1 nuclease. Lanes: 1, No RNA in the hybridization; 2, hybridization with early vaccinia RNA; 3, ^{32}P-labeled *Hae*III fragments of φX174 DNA, 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, and 118 base pairs.

employed such as distinctive molecular weight, immunological detection, affinity for specific substrates, covalent modification or peptide analysis.

In this report the identification of the gene encoding the large subunit of the capping enzyme was facilitated by the formation of a covalent enzyme-guanylate intermediate. Preliminary attempts to detect the guanylylation of the cell-free translation product were unsuccessful. Therefore, to facilitate this identification, the correspondence between the ^{32}P-labeled large subunit found in vaccinia virions and a cell-free translation product was based on molecular weight, affinity for DNA-cellulose, migration in two-dimensional gels, and partial enzymatic hydrolysis. By hybrid selection coupled with cell-free translation, the mRNA encoding the 84K polypeptide was mapped 3.1 kb to the left of the *Hin*dIII D fragment of the vaccinia genome. The precise coordinates of the RNA transcript were defined by a combination of RNA filter hybridization and S1 nuclease mapping.

The identification of the DNA sequences encoding the large subunit of the capping enzyme represents the first function required for mRNA biogenesis to be located on the vaccinia genome. This information strengthens the presumption that this virus encodes the entire complement of activities required to transcribe and process mRNAs. In addition to the large subunit, the capping enzyme contains a smaller 26K subunit, and our preliminary evidence indicates that an mRNA encoding a 26K polypeptide with affinity for DNA-cellulose is also located on the *Hin*dIII D fragment. More to the left, adjacent to the mRNA encoding the large subunit of the capping enzyme, is an mRNA encoding a 34K polypeptide which demonstrates an affinity for poly(A) agarose. This poly(A)-binding polypeptide correlates in size with one of the two subunits composing the poly(A) polymerase which is purified by affinity chromatography on poly(A) agarose (30). Adjacent to this gene is an mRNA encoding a 110K polypeptide identical in size to the large subunit of the DNA-dependent RNA polymerase (20). Taken together, these preliminary observations raise the intriguing possibility that genes encoding related functions are grouped together on the viral genome.

This region encompassing the *Hin*dIII J, H, and D fragments contains mutations coding for numerous temperature-sensitive (*ts*) mutants, none of which exhibit the expected phenotype for a virus encoding an enzyme defective in mRNA biogenesis (9). This could indicate that the regimens utilized to prepare these mutants precluded the isolation of *ts* mutants in the mRNA biogenesis functions or that the actual phenotypes of such mutants are not as expected. Perhaps this enigma can be solved by utilizing the DNA sequences encoding the large subunit of the capping enzyme in marker rescue studies to determine whether any of the *ts* mutations located in the *Hin*dIII D fragment lie within this gene.

In the event that none of these mutations are located within the gene, alternate methods for the generation and isolation of mutants should be utilized. The availability of a defined gene permits the generation of specific lesions through direct mutagenesis and reintroduction of the altered sequences into the viral genome by marker transfer. For essential genes, the growth and isolation of the defective viruses would require complementation of the wild-type function by mammalian cell lines constitutively expressing the large subunit of the capping enzyme. This approach will

permit the preparation of *ts* and null mutations to define the functional domains of the capping enzyme and to determine the physiological roles of the cap structure in mRNA transcription, translation, and stability.

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