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Biochemistry

Transcription initiation factor activity of vaccinia virus capping enzyme is independent of mRNA guanylylation

(poxvirus/gene regulation/RNA polymerase)

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ABSTRACT **Cytoplasmic extracts of vaccinia virus-infected HeLa cells blocked in DNA replication were capable of transcribing templates containing the minimal promoter sequences derived from three viral intermediate-stage genes (*A1L*, *A2L*, and *G8R*) but not promoters from early or late genes. One of three isolated components required for transcription copurified with the viral capping enzyme, a heterodimeric protein responsible for forming the 7-methylguanosine(5′)triphospho(5′)nucleoside [m^{7}G(5′)ppp(5′)N-] structure at the 5′ end of mRNAs, as had been reported using a template with another intermediate promoter [Vos, J. C., Sasker, M. & Stunnenberg, H. G. (1991) *EMBO J.* 10, 2553–2558]. Transcription factor activity was associated with partially purified capping enzyme from infected cell extracts, homogeneous enzyme from purified virions, and recombinant viral enzyme from *Escherichia coli*. By transcribing truncated templates of different sizes, we determined that RNA chains of 35 nt were capped whereas those of 15 nt were not. Nevertheless, the capping enzyme was required for formation of short uncapped transcripts, indicating that capping and transcription initiation factor activities are independent functions.**

Vaccinia virus provides a powerful system for combining biochemical and genetic approaches to the study of eukaryotic-like transcription mechanisms (1). This best-characterized member of the poxvirus family has a 200,000-bp double-stranded DNA genome that encodes catalytic and regulatory components for programmed gene expression in the cytoplasm of infected cells. The known viral proteins involved in mRNA biosynthesis include a DNA-dependent RNA polymerase of which some subunits are homologous to those of the cellular enzyme, several stage-specific transcription factors, and mRNA capping, methylating, and polyadenylylating enzymes (2). The cytoplasmic location and full complement of virus-encoded enzymes and transcription factors contribute to the independence of vaccinia virus from the nuclear transcription machinery of the cell.

Vaccinia virus genes have been grouped into early, intermediate, and late classes, according to the stage of infection in which they are activated. Expression of the early genes is mediated by proteins that are packaged within the virus particle and occurs immediately after viral entry into the cell. Approximately half of the viral genes belong to this pre-DNA replication class. Expression of the intermediate and late genes requires *de novo* viral protein synthesis as well as DNA replication.

Promoter elements of the three classes of genes have been characterized. Those belonging to the early class have an A+T-rich consensus sequence between nt −28 and −13, relative to the RNA start site (3). Only five intermediate genes have been identified thus far (4, 5), a number too small to yield a consensus sequence. Deletion and linker-scanning mutagenesis of the *I3L* gene intermediate promoter indicated two critical regions between positions −20 and −9 and between positions +1 and +9 (6). A finer mutational analysis of the promoters associated with three other intermediate genes (*A1L*, *A2L*, and *G8R*) defined an A + T-rich core element at positions −26 to −13 and a TAAA sequence initiator element at positions −1 to +3 of the nontemplate strand (7). The promoter elements of late genes include a TAAAT initiator sequence and upstream thymidine residues in the nontemplate strand (8).

Virus-encoded stage-specific transactivating factors exist for each class of genes. These factors are expressed in a sequential fashion forming a regulatory cascade. Thus, early proteins are required for intermediate transcription, intermediate proteins are required for late transcription and late proteins packaged into virus particles are required for early transcription. Genes encoding early (9–11) and late (5, 12) transcription factors have been identified. The most recent additions to the regulatory cascade are transcription factors specific for intermediate genes. *In vitro* studies using an *I3L* promoter template led to the separation of two fractions that, in combination with vaccinia RNA polymerase, were necessary and sufficient for *in vitro* transcription (13). One partially purified factor, called vaccinia virus intermediate transcription factor (VITF) B, mediated promoter melting; the other called VITF A copurified with viral capping enzyme activity (14). Capping enzyme was involved in the formation of a stable initiation complex, a surprising observation since this enzyme is not required for transcription of early genes, although it does have a role in the termination process (15, 16). There is also no evidence that cellular capping enzyme has a role in transcription mediated by RNA polymerase II (17, 18).

The steps involved in mRNA capping were originally determined for the vaccinia virus heterodimeric enzyme complex that has mRNA triphosphatase, mRNA guanylyltransferase, and mRNA (guanine-N^7-)-methyltransferase activities (19–22). An initial step in cap formation is the removal of the γ-phosphate from the triphosphate end of the nascent mRNA; a covalent intermediate is formed between a GMP residue derived from GTP and the 95-kDa subunit of the capping enzyme; this GMP is transferred to the 5′-diphosphate end of the mRNA to form a unique 5′-5′ triphosphate bond; and finally, a methyl group is transferred from *S*-adenosylmethionine to the N7 position of the 5′-guanosine moiety to create the 7-methylguanosine(5′)triphospho(5′)nucleoside [m^{7}G(5′)ppp(5′)N-] cap structure. Similar steps are involved in the capping of eukaryotic mRNA, although the guanylyl- and methyltransferase activities are present in separate proteins. Vos *et al.* (14) were unable to determine whether the above activities of the vaccinia virus capping

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Abbreviations: araC, cytosine arabinoside; CCE, cytoplasmic cell extract; VITF, vaccinia virus intermediate transcription factor; DTT, dithiothreitol.

enzyme were required for the stimulation of transcription or whether the latter was an entirely separate function.

In the study reported here, we obtained evidence for the general role of the vaccinia virus capping enzyme in intermediate transcription by using templates containing the *A1L*, *A2L*, and *G8R* promoters. Furthermore, we demonstrated that capping of intermediate transcripts occurs after the RNA chain is longer than 15 nt. Nevertheless, capping enzyme was required for the formation of short uncapped transcripts as well as longer capped ones. Thus, the role of capping enzyme as a transcription initiation factor is independent of its role in capping.

MATERIALS AND METHODS

Preparation and Fractionation of Infected-Cell Extracts. HeLa S3 cells were grown in suspension in Eagle's medium modified for spinner culture and supplemented with 5% (vol/vol) horse serum. The cells, at 5×10^5 cells per ml, were incubated with cytosine arabinoside (araC; 40 μg/ml; Sigma) starting 2 h prior to infection. The cells were then concentrated 40-fold and inoculated with vaccinia virus (WR strain) at a multiplicity of 10 plaque-forming units per cell. After stirring for 30 min at 37°C, the infected cells were collected by centrifugation and resuspended at 5×10^5 cells per ml in fresh araC-containing medium. The infected cells were harvested after incubation for 16 h at 37°C and Dounce-homogenized, and the nuclei were removed by centrifugation at 14,500 × *g* for 15 min. A soluble extract was prepared from the cytoplasmic fraction as described by Manley (23) with minor modifications. Protein was precipitated from the 175,000 × *g* supernatant by addition of $(NH_4)_2SO_4$ to a final concentration of 2 M. The precipitate was resuspended and dialyzed against DEI buffer [40 mM Tris·HCl, pH 7.9/0.25 M KCl/0.2 mM EDTA/2 mM dithiothreitol (DTT)/15% (vol/vol) glycerol]. The extract from 5×10^9 cells was applied to a 10 cm (length) × 3 cm (diameter) DEAE-cellulose column (DE52, Whatman) equilibrated with DEI buffer containing 0.25 M KCl. The flow through was dialyzed against DEI buffer containing 0.1 M KCl and is referred to as the cytoplasmic cell extract (CCE). The CCE was applied to a second DEAE-cellulose column (10 cm × 3 cm). The flow through, containing VITF A, was collected, and the column was eluted with DEI buffer containing 0.25 M KCl. The eluate was dialyzed against DEI buffer containing 0.1 M KCl and then applied to a 4 cm × 3 cm phosphocellulose (P11, Whatman) column. A 400-ml linear 0.1–0.6 M KCl gradient was applied. The column fractions were tested in RNA polymerase and transcription assays as described below. RNA polymerase and VITF B were eluted at 0.36–0.4 M KCl and 0.57–0.6 M KCl, respectively. Where indicated, RNA polymerase and VITF B were separated by chromatography on a 10 cm × 3 cm DEAE-cellulose column, which was developed with a 500-ml linear 0.1–0.6 M KCl gradient. Under these conditions, RNA polymerase and VITF B were eluted at 0.16–0.19 and 0.21–0.24 M KCl, respectively.

Transcription Assays. Transcription reactions were routinely carried out at 30°C for 30 min by using CCE or reconstituted fractions in total volumes of 50 μl or 100 μl, respectively, containing 60 mM KCl, 0.14 mM EDTA, 20 mM Tris·HCl (pH 7.9), 2 mM $MgCl_2$, 2 mM ATP, 0.2 mM CTP, 0.02 mM UTP, 5 μCi of [α-^{32}P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq), 0.1 μg of DNA template, and 4% (vol/vol) polyvinyl alcohol. Under standard conditions, a supercoiled plasmid containing a G-less cassette template was employed, RNase T1 (25–50 units, Boehringer Mannheim) was added at the end of the transcription reaction, and the mixture was incubated further at 37°C for 15 min. Reaction stop buffer (250 μl; 8 M urea/10 mM Tris·HCl/10 mM EDTA/0.5% SDS) was then added. The RNA was phenol/chloroform-extracted, ethanol-precipitated, and analyzed on a 4% gel [acrylamide/*N*,*N*′-methylenebisacrylamide, 20:1 (wt/wt)] containing 8 M urea and 1× TBE (24).

Truncated linear DNA templates (0.1 μg) were transcribed with the following modifications. For internal labeling, the 100-μl reaction mixtures contained 0.2 mM UTP, 0.02 mM CTP, 20 μCi of [α-^{32}P]CTP (3000 Ci/mol), 0.06 μM GTP, and 50 μM *S*-adenosyl-L-methionine. For cap labeling, the reaction mixtures contained 0.2 mM UTP, 0.02 mM CTP, 40 μCi of [α-^{32}P]GTP (3000 Ci/mmol), and 50 μM *S*-adenosyl-L-methionine. Reaction stop buffer (200 μl) was used to terminate transcription, and RNA was extracted as above and analyzed on a denaturing 20% polyacrylamide gel.

DNA Templates. The minimal essential promoter sequences of intermediate genes *G8R*, *A1L*, and *A2L* (7) were cloned separately into a G-less cassette vector, $(C_2AT)19$ (25), and used as templates in the standard transcription assays. PCR was used to prepare the *G8R* promoter fragment (nt −29 to +6) ATTTAACTTTAAATAATTTACAAA-AATTTAAAATA contiguous with either AATTCCCCC-GGG or AATTCCATACCCTTCCTCCATCTATAC-CCGGG ending in *Sma* I sites. The PCR products were cloned into the "insertion site" of the TA cloning vector plasmid (Invitrogen). DNA fragments of ≈700 bp were excised from the plasmids with *Hin*dIII and *Sma* I. The fragments were gel-purified and used as templates for transcription reactions that formed 15- and 35-nt RNAs that start with adenosine, lack guanosine residues at internal positions, and end with cytidine.

Purification of Capping Enzyme. Purified vaccinia virus (2×10^{13} particles) was suspended in 20 ml of buffer A (0.25 M NaCl/0.1 M Tris·HCl, pH 8.4/0.1 mM EDTA/10 mM DTT). Sodium deoxycholate was added to 0.2% and the suspension was incubated on ice for 1 hr. After Dounce homogenization, the viral suspension was centrifuged for 30 min at 14,470 × *g* at 4°C. The supernatant was removed and extraction of the viral pellet was repeated. The combined supernatants were applied to a 14 cm × 1.5 cm DEAE-cellulose (DE52, Whatman) column equilibrated with buffer A. The flow through containing capping enzyme was dialyzed against buffer B (80 mM NaCl/50 mM Tris·HCl, pH 8/0.1 mM EDTA/1 mM DTT/10% glycerol/0.01% Nonidet P-40) and then applied to another DEAE-cellulose column of 8.5 cm × 1.5 cm. The flow through, containing capping enzyme, was chromatographed on a 6 cm × 1 cm heparin-agarose (GIBCO/BRL) column developed with a 150-ml linear 0.08–1 M NaCl gradient. Capping enzyme was eluted at 0.3 M NaCl and was applied to a 6 cm × 1 cm phosphocellulose (P11, Whatman) column in buffer B at 80 mM NaCl. The column was developed with a 75-ml linear salt gradient from 0.08 to 0.5 M NaCl. Capping enzyme was eluted at 0.18 M NaCl and was applied to a 6 cm × 1 cm single-stranded DNA-agarose column (BRL) in buffer B at 30 mM NaCl. Protein was chromatographed with a 75-ml linear salt gradient from 0.03 to 0.45 M NaCl. Capping enzyme was eluted at 0.13 M NaCl and was applied to a 3 cm × 1 cm diameter Q-Sepharose column (Pharmacia) in buffer B. The column was developed with a 20-ml linear salt gradient from 0 to 0.5 M NaCl and capping enzyme was eluted at 0.17 M NaCl.

RNA Polymerase Assay. RNA polymerase was assayed as described (26) in 80 mM NaCl/135 mM Tris·HCl, pH 8/17 mM DTT/7 mM $MnCl_2$/2 mM ATP/2 mM CTP/2 mM GTP/0.2 mM UTP/2 μCi of [α-^{32}P]UTP/1 μg of M13mp18 single-stranded DNA at 37°C for 20 min.

Capping Enzyme Assay. Capping enzyme was assayed by the formation of an enzyme–guanylate complex in 50 mM Tris·HCl, pH 8.2/2 mM DTT/5 mM $MgCl_2$/2 μCi of [α-^{32}P]GTP at 37°C for 10 min (27). The reaction was terminated with 1% SDS and the complex was analyzed by SDS/PAGE and autoradiography.

RESULTS

***In Vitro* Transcription of Vaccinia Virus Intermediate Promoter Templates.** Previous studies indicated that plasmids containing reporter genes regulated by the viral *A1L, A2L,* and *G8R* minimal promoters were transcribed upon their transfection into cells infected with vaccinia virus in the presence of araC, an inhibitor of DNA replication (7). The same promoters, cloned adjacent to a sequence lacking guanosine residues in the coding strand (G-less cassette; ref. 25) of a plasmid, were used as templates to assess the *in vitro* transcriptional activity of cytoplasmic extracts prepared from infected and uninfected araC-treated HeLa cells. The infected, but not the uninfected, extract was able to transcribe each of the templates (Fig. 1). The G-less cassette without a promoter (Fig. 1), cassettes containing vaccinia virus early and late promoters (data not shown), and a *G8R* cassette with deleterious single point mutations in the core region (7) of the promoter (data not shown) were not transcribed by the infected-cell extract.

Infected araC-treated HeLa cells provided the starting material to purify intermediate transcription factors. The procedure outlined in Fig. 2*A* was used to obtain RNA polymerase and transcription factor fractions, called VITF A and VITF B according to Vos *et al.* (13). The three fractions were necessary and sufficient to transcribe the *G8R* promoter G-less cassette template (Fig. 2*B*).

Copurification of Capping Enzyme and VITF A. By using the *I3L* gene promoter as template, the transcription stimulatory activity of VITF A was found to copurify with viral capping enzyme activity (14). To evaluate the role of capping enzyme in transcription of other intermediate promoters, the VITF A fraction from cytoplasmic extracts (Fig. 2*A*) was chromatographed on heparin-agarose. Transcription activity in the column fractions, assayed with the *G8R* promoter-containing template in the presence of VITF B and RNA polymerase, coeluted with capping enzyme activity measured by formation of a covalent guanylate complex with the 95-kDa subunit (Fig. 3*A*). Moreover, heparin-agarose fractions of capping enzyme extracted from purified vaccinia virions also coeluted with transcription stimulatory activity (Fig. 3*B*). Transcription activity from virion extracts copurified with capping enzyme over five columns until the latter appeared homogeneous, as determined by detection of silver-stained 95- and 31-kDa subunits (Fig. 4). Furthermore, purified recombinant viral capping enzyme expressed in *Escherichia coli* (28) had transcriptional stimulatory activity (Fig. 4*C*).

Purified capping enzyme was required for transcription of templates regulated by the *A1L* and *A2L* promoters (data not shown), as well as the *G8R* promoter, indicating that it serves as a transcription factor for all tested intermediate genes.

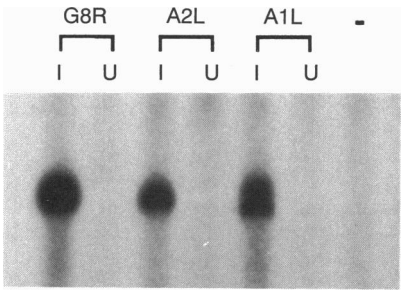

FIG. 1. Intermediate gene transcription by an infected-cell extract. Cytoplasmic extracts made from infected araC-treated HeLa cells (lanes I) and uninfected cells (lanes U) were assayed using intermediate gene promoter (*G8R, A1L,* and *A2L*) G-less cassette DNA templates. Lane − indicates a promoterless template. Radioactively labeled RNA was resolved on a denaturing polyacrylamide gel and analyzed by autoradiography.

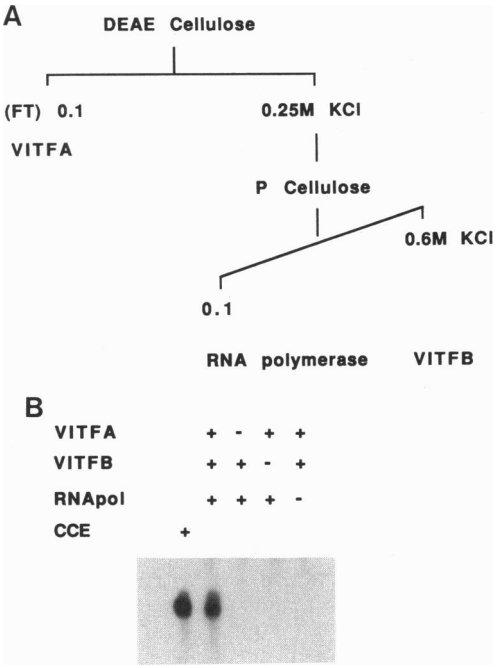

FIG. 2. Reconstitution of transcription activity. (*A*) Scheme for separation of transcription components VITF A, RNA polymerase, and VITF B by DEAE-cellulose and phosphocellulose (*P*) column chromatography of a cytoplasmic extract from vaccinia virus-infected araC-treated HeLa cells. FT, flow through. (*B*) Transcription activity of the CCE and reconstituted column fractions containing RNA polymerase (pol), VITF A, and VITF B. An intermediate gene promoter (*G8R*) G-less cassette DNA template was used. Radioactively labeled RNA was resolved by polyacrylamide gel electrophoresis and detected by autoradiography.

RNA Capping Is Not Required for Transcription Factor Activity. The requirement of vaccinia virus capping enzyme

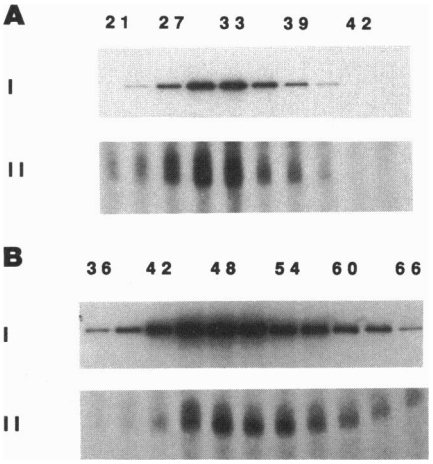

FIG. 3. Cochromatography of capping enzyme and transcription-stimulatory activities. (*A*) The DEAE-cellulose flow-through fraction from an infected-cell cytoplasmic extract (VITF A, Fig. 2) was chromatographed on heparin-agarose with a linear salt gradient of 0.08–1.0 M NaCl. Every third column fraction was assayed for capping activity by formation of a 95-kDa covalent GMP intermediate (bands I) and for transcription factor activity with added DEAE-cellulose-purified RNA polymerase and VITF B (bands II). (*B*) The DEAE-cellulose flow-through fraction from a virion extract was chromatographed on heparin-agarose and assayed for capping (bands I) and transcription factor (bands II) activities. Column fraction numbers are indicated above the autoradiographs.

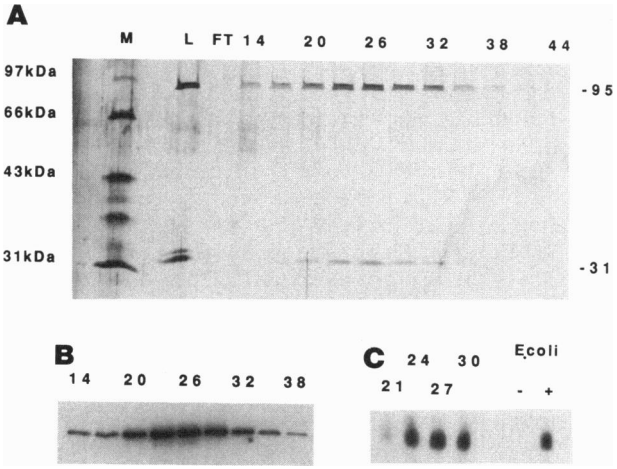

FIG. 4. Transcription factor activity of purified and recombinant capping enzyme. (*A*) Capping enzyme was extracted from virions and chromatographed on five successive columns. Every third fraction of the Q-Sepharose column was analyzed by SDS/PAGE and silver staining. Lanes: M, standard protein markers; L, pooled fractions from the DNA-agarose column loaded on to Q-Sepharose; FT, Q-Sepharose flow-through fraction; numbers, column fractions. The positions of the 95- and 31-kDa subunits are indicated on the right; positions of standard protein markers are shown on the left. (*B*) Fractions from the Q-Sepharose column of *A* were assayed for capping enzyme activity as in Fig. 2. (*C*) Transcription factor activity of fractions from the Q-Sepharose column and recombinant capping enzyme made in *Escherichia coli*. Column fractions 21–23, 24–26, 27–30, and 30–33 of *A* were pooled and assayed for transcription activity with added RNA polymerase and VITF B as in Fig. 3. Recombinant vaccinia virus capping enzyme from Guo and Moss (28) was assayed for transcription factor activity. Lane labels indicate the number of the first fraction of each pool. − and +, Omission and addition of recombinant capping enzyme, respectively.

for intermediate transcription could be explained if RNA cap formation was an integral part of the transcription initiation process. Seemingly, a simple way of testing this hypothesis would be to use a G-less cassette template and omit GTP from the transcription reaction. However, even when ultra-pure ATP, CTP, and UTP were used, there was sufficient contaminating GTP to load capping enzyme with a GMP residue (14). We decided, therefore, to directly determine the stage at which capping of intermediate transcripts occurred. Previous studies had shown that RNA polymerase II transcripts (18) as well as vaccinia virus early mRNAs (29) are not capped until they are between 20 and 30 nt long, consistent with the lack of capping enzyme requirements for transcription initiation in those cases. To determine when capping of intermediate mRNAs occurred, a series of truncated linear DNA templates were designed to allow synthesis of run-off RNAs from 10 to 35 nt long. Each template had a *G8R* promoter attached to a sequence devoid of cytidine residues in the noncoding strand to preclude the incorporation of complementary guanosine residues by RNA polymerase. Under these conditions, [α-^{32}P]GTP could only be incorporated into the 5′-terminal cap structure whereas [α-^{32}P]CTP would be incorporated into internal positions in the RNA chain. Preliminary results indicated that transcripts of 20 nt or less were labeled with CTP but not GTP, whereas longer transcripts were labeled with both precursors. The results obtained with templates for RNAs of 15 and 35 nt are shown. With the short template, a series of CTP-labeled bands estimated to be 13–17 nt long were resolved (Fig. 5*A*). The multiplicity of the bands could be due to heterogeneous intitiation within the TAAAT sequence, RNA polymerase slippage, or premature termination. Significantly, none of these bands was labeled with GTP (Fig. 5*A*). Nevertheless, capping enzyme (VITF A) was required for transcription despite the absence of RNA capping (Fig. 5*B*). In contrast, transcripts of ≈35 nt were efficiently labeled with both CTP and GTP (Fig. 6). Again, capping enzyme was required for transcription.

DISCUSSION

By testing additional intermediate promoters, namely, those that regulate three transactivators of late gene expression, we have confirmed and extended the finding of Vos *et al.* (14) that vaccinia virus capping enzyme serves as a stage-specific transcription factor. Identical results were obtained with the viral capping enzyme partially purified from the cytoplasm of infected cells, homogeneous enzyme purified from virions, and recombinant viral enzyme made by *E. coli*. Most significantly, we demonstrated that the mechanism of transcription stimulation does not involve capping of the mRNA but represents yet another activity of this versatile enzyme.

The vaccinia virus capping enzyme is a heterodimer of 95- and 31-kDa subunits (30). Both subunits are encoded within the viral genome and are expressed during the early phase of infection (31, 32). The enzymatic activities related to cap formation include RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-N^7-)-methyltransferase (21). RNA triphosphatase and guanylyltransferase are associated with the 95-kDa subunit (28, 33), whereas both subunits are required for methyltransferase activity (34, 35). An indication that the capping enzyme might have additional roles in mRNA biosynthesis came from its copurification with a transcription termination factor activity (15, 36). Unlike the capping function, which occurs with transcripts of all three

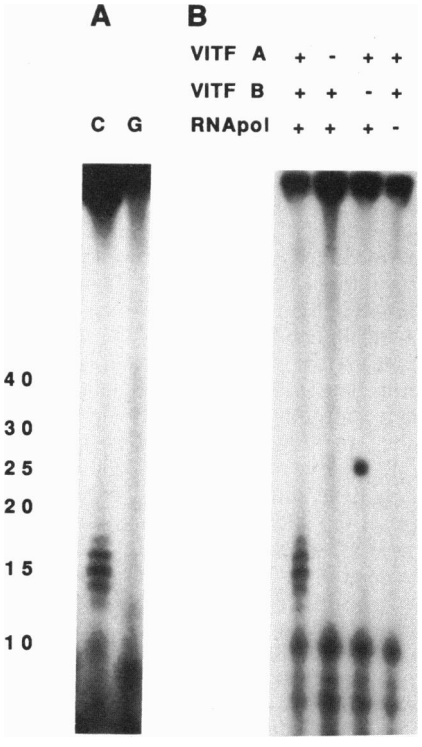

FIG. 5. Transcription factor requirements for synthesis of 15-nt uncapped RNA. (*A*) An intermediate *G8R* promoter regulated, linear, G-less template was truncated 15 bp downstream of the RNA start site and transcribed with VITF A, VITF B, and RNA polymerase in the presence of [α-^{32}P]CTP (lane C) or [α-^{32}P]GTP (lane G). The transcripts were analyzed by polyacrylamide gel electrophoresis and autoradiography. Sizes in nucleotides are shown to the left. (*B*) Transcription activities of combinations of VITF A, VITF B, and RNA polymerase were determined in the presence of [α-^{32}P]CTP as described in *A*. (The spot over the VITF A +/VITF B −/RNA pol + lane at the 25-nt position is an artifact.)

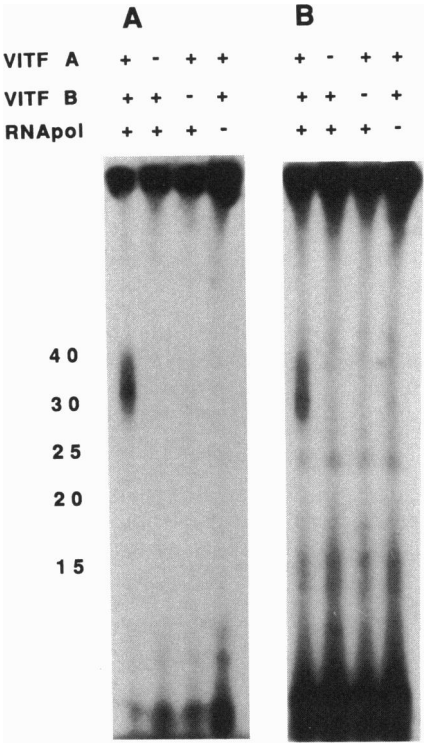

FIG. 6. Transcription factor requirements for synthesis of 35-nt capped RNA. Combinations of VITF A, VITF B, and RNA polymerase were used to transcribe a template similar to that described in Fig. 5 except that the template was truncated 35 bp downstream of the RNA start site. (*A*) [α-^{32}P]CTP. (*B*) [α-^{32}P]GTP. Sizes in nucleotides are shown to the left.

classes of viral genes, termination factor activity is specific for early genes and involves the recognition of the sequence UUUUUNU in the nascent mRNA (14–16). Transcription initiation factor activity of capping enzyme is also stage-specific since it is required with intermediate but not early promoters. Whether capping enzyme is required as an initiation factor for late genes has not yet been established.

The possibilities that capping and transcription initiation are coupled or that the presence of a cap on short oligonucleotides contributes to the stability of the transcription complex seemed the most obvious reasons for a capping enzyme requirement. In this regard, dinucleotides such as ppGpC and even the mononucleotides ppG and ppA can be capped, albeit inefficiently, by the purified vaccinia virus enzyme (20). To investigate such mechanisms, intermediate promoter-regulated linear templates truncated 15 or 35 bp downstream of the site of RNA initiation were constructed. Despite the short length of DNA downstream of the RNA start site available for RNA polymerase binding, both templates were transcribed. However, only the transcripts made with the longer template were capped. Nevertheless, capping enzyme was absolutely required for formation of short uncapped transcripts leading to the inescapable conclusion that capping and transcription factor activities are independent functions of the vaccinia virus capping enzyme. Similarly, early transcription termination factor activity is not dependent on RNA capping and also represents an independent function (15). The association of capping enzyme with RNA polymerase (14, 37, 38) is a feature that would seem to be critical for all of its roles.

Since short RNAs can be capped by purified capping enzyme (20), the length requirement for capping of intermediate transcripts is likely to have a steric basis. Presumably, the 5′ end of the nascent RNA must emerge from the catalytic site of the polymerase before capping enzyme gains access to it. Similar chain-length requirements for capping have been found for RNA polymerase II transcripts (18) and for vaccinia virus early mRNAs (29).

The capping enzyme is the only defined component of the reconstituted vaccinia virus intermediate gene transcription system. The nature of VITF B and the gene(s) encoding this factor have not been reported. In addition, the subunit structure of the RNA polymerase synthesized prior to DNA replication and used for transcription of intermediate genes needs to be examined and the subunits need to be compared with those of the virion-associated RNA polymerase used for transcription of early genes.

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1. Moss, B. (1990) *Annu. Rev. Biochem.* **59,** 661–688.
2. Moss, B., Ahn, B.-Y., Amegadzie, B. Y., Gershon, P. D. & Keck, J. G. (1991) *J. Biol. Chem.* **266,** 1355–1358.
3. Davison, A. J. & Moss, B. (1989) *J. Mol. Biol.* **210,** 749–769.
4. Vos, J. C. & Stunnenberg, H. G. (1988) *EMBO J.* **7,** 3487–3492.
5. Keck, J. G., Baldick, C. J. & Moss, B. (1990) *Cell* **61,** 801–809.
6. Hirschmann, P., Vos, J. C. & Stunnenberg, H. G. (1990) *J. Virol.* **64,** 6063–6069.
7. Baldick, C. J., Keck, J. G. & Moss, B. (1992) *J. Virol.* **66,** 4710–4719.
8. Davison, A. J. & Moss, B. (1989) *J. Mol. Biol.* **210,** 771–784.
9. Broyles, S. S. & Fesler, B. S. (1990) *J. Virol.* **64,** 1523–1529.
10. Gershon, P. D. & Moss, B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4401–4405.
11. Ahn, B.-Y. & Moss, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3536–3540.
12. Wright, C. F., Keck, J. G. & Moss, B. (1991) *J. Virol.* **65,** 3715–3720.
13. Vos, J. C., Saskar, M. & Stunnenberg, H. G. (1991) *Cell* **65,** 105–114.
14. Vos, J. C., Sasker, M. & Stunnenberg, H. G. (1991) *EMBO J.* **10,** 2553–2558.
15. Shuman, S., Broyles, S. S. & Moss, B. (1987) *J. Biol. Chem.* **262,** 12372–12380.
16. Shuman, S. & Moss, B. (1988) *J. Biol. Chem.* **263,** 6220–6225.
17. Bunick, D., Zandomeni, R., Ackerman, S. & Weinmann, R. (1982) *Cell* **29,** 877–886.
18. Coppola, J. A., Field, A. S. & Luse, D. S. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1251–1255.
19. Martin, S. A. & Moss, B. (1975) *J. Biol. Chem.* **250,** 9330–9335.
20. Martin, S. A. & Moss, B. (1976) *J. Biol. Chem.* **251,** 7313–7321.
21. Venkatesan, S., Gershowitz, A. & Moss, B. (1980) *J. Biol. Chem.* **255,** 903–908.
22. Shuman, S. & Hurwitz, J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 187–191.
23. Manley, J. L. (1987) in *Transcription and Translation: A Practical Approach*, eds. Hames, B. D. & Higgins, S. J. (IRL, Oxford), pp. 72–73.
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Plainview, NY), 2nd Ed., p. 454.
25. Sawadogo, M. & Roeder, R. G. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4394–4398.
26. Baroudy, B. M. & Moss, B. (1980) *J. Biol. Chem.* **255,** 4372–4380.
27. Shuman, S. & Moss, B. (1990) *Methods Enzymol.* **181,** 170–180.
28. Guo, P. & Moss, B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4023–4027.
29. Hagler, J. & Shuman, S. (1992) *J. Virol.* **66,** 2982–2989.
30. Martin, S. A., Paoletti, E. & Moss, B. (1975) *J. Biol. Chem.* **250,** 9322–9329.
31. Morgan, J. R., Cohen, L. K. & Roberts, B. E. (1984) *J. Virol.* **52,** 206–214.
32. Niles, E. G., Lee-Chen, G.-J., Shuman, S., Moss, B. & Broyles, S. S. (1989) *Virology* **172,** 513–522.
33. Shuman, S. & Morham, S. G. (1990) *J. Biol. Chem.* **265,** 11967–11972.
34. Cong, P. & Shuman, S. (1992) *J. Biol. Chem.* **267,** 16424–16429.
35. Higman, M. A., Bourgeois, N. & Niles, E. G. (1992) *J. Biol. Chem.* **267,** 16430–16437.
36. Luo, Y., Hagler, J. & Shuman, S. (1991) *J. Biol. Chem.* **266,** 13303–13310.
37. Broyles, S. S. & Moss, B. (1987) *Mol. Cell. Biol.* **7,** 7–14.
38. Hagler, J. & Shuman, S. (1991) *Science* **255,** 983–986.