The EMBO Journal vol.10 no.9 pp.2553-2558, 1991

Vaccinia virus capping enzyme is a transcription initiation factor

Jan C.Vos[1], Menno Sasker and Hendrik G.Stunnenberg

European Molecular Biology Laboratory, Gene Expression Program, Meyerhofstrasse 1, 6900 Heidelberg, FRG
[1]Present address: Dutch Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Communicated by I.W.Mattaj

It has previously been demonstrated that vaccinia virus capping enzyme is involved both in the formation of a 5′ cap structure and in termination of early transcription. Here we show that capping enzyme has an additional activity which is required for transcription of intermediate genes. VITF-A and VITF-B have been defined as two activities which together with RNA polymerase are necessary and sufficient to transcribe intermediate genes *in vitro*. VITF-A and the viral capping enzyme are shown to copurify to near homogeneity. Direct evidence that capping enzyme is VITF-A was obtained by complementation of a reconstituted transcription system with viral capping enzyme expressed in *Escherichia coli*. Although capping enzyme is a cofactor in early transcription termination, intermediate transcription is not terminated in response to the early termination signal. Capping enzyme is shown to form a complex with RNA polymerase in the absence of VITF-B. This appears to be a prerequisite for the formation of a stable initiation complex.

Key words: capping enzyme/vaccinia virus/termination/transcription initiation factor

Introduction

The synthesis of mature mRNA in eukaryotic cells is a multi-step process involving transcription initiation, elongation and termination, splicing and RNA modifications. The development of cell-free systems has enabled the uncoupling of several of these steps and allowed them to be studied individually. Vaccinia virus, the archetypal poxvirus, provides a unique model system for studying RNA synthesis. Proteins involved in transcription initiation, the formation of a 5′ cap structure, transcription termination as well as 3′ poly(A) tailing are of viral origin, as a consequence of the cytoplasmic site of viral replication. Viral transcription appears to be controlled in a cascade-like manner. Each class of genes (early, intermediate and late) encodes one or more factors which are required for the onset of transcription of the subsequently expressed class of genes. In addition, DNA replication is essential to switch from early to intermediate gene expression (see Moss, 1990 for review).

The virus particle contains all the proteins involved in early RNA synthesis, such as the multi-subunit RNA polymerase, capping enzyme, poly(A) polymerase and an early transcription factor. One of the first viral proteins characterized was capping enzyme, a heterodimer of a large subunit of 95 kDa and a small subunit of 31 kDa (Martin *et al.*, 1975). Three activities involved in the 5′ cap structure are associated with the protein: RNA triphosphatase, RNA guanylyltransferase and RNA (guanine-7)-methyltransferase. An enzyme–guanylate intermediate consisting of the large subunit and a covalently linked GMP residue has been described (Shuman and Hurwitz, 1981). The genes for both subunits have been identified (Morgan *et al.*, 1984; Niles *et al.*, 1989) and the protein has been expressed in *E.coli* and shown to be functional with respect to its capacity to form a 5′ cap structure (Guo and Moss, 1990). Initiation of early transcription depends solely on vaccinia virus early transcription factor (VETF) and RNA polymerase (Broyles *et al.*, 1988). Capping enzyme is not required for initiation of early transcription, but is required for the recognition of the termination signal, UUUUUNU, in the nascent RNA chain (Shuman *et al.*, 1987; Shuman and Moss, 1989). Termination occurs 20–50 nucleotides downstream of this signal.

The intermediate class of vaccinia virus genes plays a key role in the temporal regulation of gene expression (Vos and Stunnenberg, 1988). Expression of intermediate genes, and as a consequence that of late genes, is strictly dependent on DNA replication, although the factors which positively regulate intermediate transcription are present within the cell prior to replication (Vos and Stunnenberg, 1988). An *in vitro* system for studying intermediate transcription has been developed and has resulted in the identification of two activities, called VITF-A and VITF-B, which together with the viral RNA polymerase are necessary and sufficient to direct intermediate transcription. VITF-B confers intermediate promoter specificity to an early promoter-specific extract prepared from virus particles. Binding of VITF-B to the promoter results in formation of a committed complex which is resistant to challenge with a second template. In the presence of ATP, VITF-A and RNA polymerase are recruited by the template-committed complex into an initiation complex which is heparin resistant. Finally, VITF-B appears to be the sole factor required for promoter melting, a process which is stimulated by ATP (Vos *et al.*, 1991).

Here, we report on the purification and identification of VITF-A as the viral capping enzyme. Experiments to define the role of capping enzyme in intermediate transcription suggest that it has a structural role in the formation of an initiation complex.

Results

Purification of VITF-A

VITF-A has been defined as an activity which, in addition to VITF-B and RNA polymerase, is required for the transcription of intermediate genes *in vitro* (Vos *et al.*, 1991).

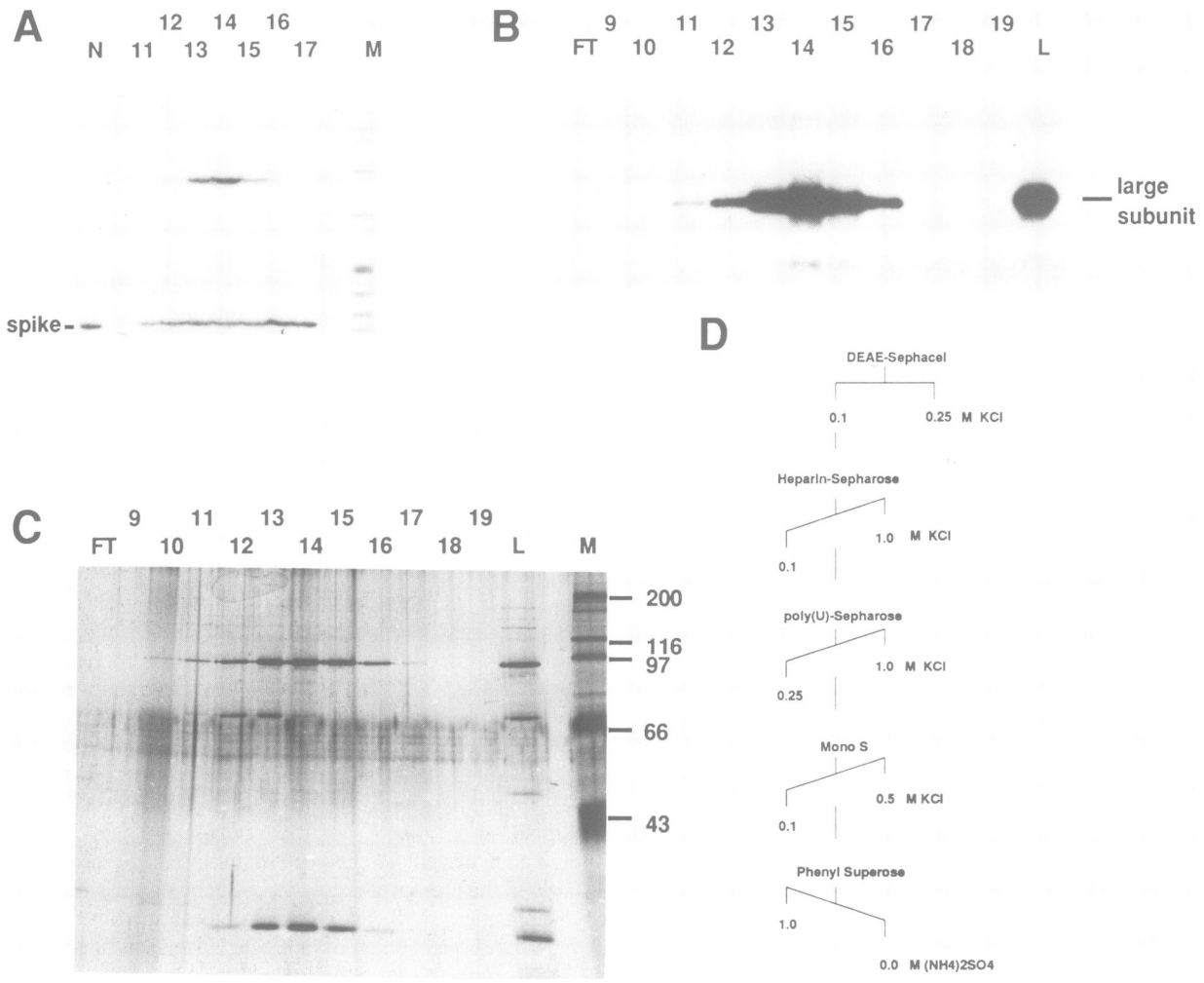

Fig. 1. Purification and analysis of VITF-A. (**A**) *In vitro* transcription complementation assay. Phenyl-Superose column fractions (0.5 μl) were used to complement RNA polymerase (0.2 μl) and VITF-B (0.2 μl) in a reconstituted transcription system using 250 ng of pIΔG[380] as template. RNA products were separated on a sequencing gel. Lane N shows transcription in the absence of VITF-A. Radiolabelled pBR322 *Hpa*II fragments were used as markers (lane M). (**B**) Transguanylylation assay. The large subunit of capping enzyme was labelled with [α-^{32}P]GTP in a transguanylylation assay. The enzyme–guanylate intermediate was electrophoresed on a 10% SDS–polyacrylamide gel. An autoradiograph of the gel is shown. FT and L stand for flowthrough and load, respectively. (**C**) SDS–PAGE. Silver-stained 10% SDS–polyacrylamide gel of the phenyl-Superose fractions. Molecular weight markers are indicated in kDa (lane M). (**D**) Schematic representation of the purification of VITF-A from HeLa cells infected with vaccinia virus. Column fraction numbers are indicated.

We purified VITF-A from a cytoplasmic extract prepared from HeLa cells infected with vaccinia virus. The extract was first fractionated on DEAE–Sephacel (see Figure 1D). The flowthrough fraction contained the majority of VITF-A activity, whereas VITF-B and RNA polymerase were exclusively found in the 0.25 M elution step. The flowthrough was then chromatographed on a heparin–Sepharose column. Because of co-elution of considerable amounts of capping enzyme with VITF-A on the heparin–Sepharose column, we introduced a poly(U)–Sepharose column to separate the two activities. Capping enzyme has been identified as cofactor in early transcription termination where its action depends on recognition of the RNA motif UUUUUNU (Shuman and Moss, 1989). Surprisingly, the elution patterns of VITF-A and capping enzyme appeared to be similar (data not shown). Co-elution was further observed using Mono S and phenyl-Superose chromatography. Analysis of the fractions eluted from the phenyl-Superose column is shown in Figure 1. VITF-A activity was detected in a reconstituted transcription assay, in which RNA polymerase and VITF-B are not sufficient to give rise to intermediate transcripts (Figure 1A, lane N). Complementation with VITF-A eluted from the phenyl-Superose column shows that the vast majority of VITF-A activity is recovered in fractions 13, 14 and 15. The band marked 'spike' is an internal control for recovery of RNA after stopping the transcription reaction. The presence of capping enzyme in the fractions was determined by formation of an enzyme–guanylate complex, in which [α-^{32}P]GTP is covalently linked to the large subunit (Shuman and Hurwitz, 1981). Following electrophoresis on a 10% SDS–polyacrylamide gel, the large subunit can be visualized by autoradiography. Capping enzyme is found predominantly in fractions 13, 14 and 15 (Figure 1B). Finally, the polypeptide composition of the phenyl-Superose column fractions was analysed by SDS–PAGE and silver staining. Three polypeptides of ~95, 68 and 31 kDa are detectable in the fractions containing VITF-A activity

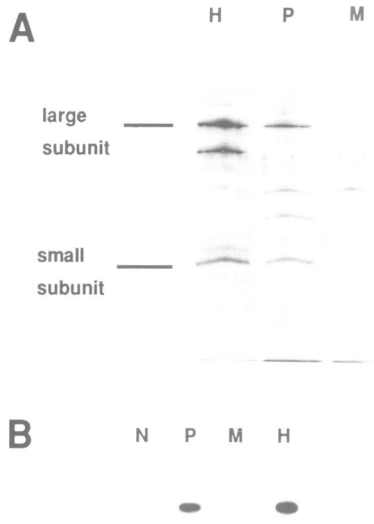

Fig. 2. Expression of capping enzyme in *E.coli*. (**A**) SDS−PAGE. Coomassie blue stained 10% SDS−polyacrylamide gel of partially purified capping enzyme from HeLa cells infected with vaccinia virus [lane H, poly(U)−Sepharose fraction] or phenyl-Superose fraction from extracts derived from *E.coli* transformed with pETD12D1 (lane P) or non-transformed bacteria (lane M). (**B**) *In vitro* transcription complementation. Complementation of RNA polymerase (0.2 μl) and VITF-B (0.2 μl) in a reconstituted transcription assay with the different fractions (1.0 μl) containing capping enzyme using 250 ng of pIΔG[380] as template. Template and protein fractions were preincubated in the presence of 1 mM ATP. After 10 min at 30°C, heparin (20 μg/ml final concentration) and nucleotides were added. RNA products were separated on a sequencing gel. Lane N shows transcription in the presence of VITF-B and RNA polymerase only.

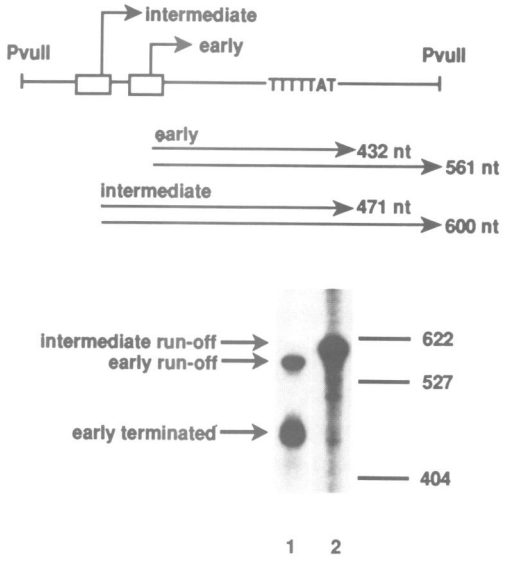

Fig. 3. Transcription termination. Two microlitres of a virion extract (lane 1) or a reconstituted (0.2 μl VITF-B, 0.2 μl RNA polymerase and 1.0 μl VITF-A) intermediate extract (lane 2) was used to transcribe the 1.1 kb *Pvu*II fragment of pI3TS (100 ng), which is schematically represented in the top of the figure. Specifically initiated and terminated early transcripts, as well as early and intermediate run-off transcripts are indicated. The position and length (bp) of DNA markers are indicated.

(Figure 1C). The 95 and 31 kDa polypeptides are characteristic of the viral capping enzyme (Martin *et al.*, 1975) and copurify with VITF-A activity, whereas the 68 kDa polypeptide peaks in fractions 12 and 13. In summary, VITF-A copurifies with capping enzyme during five chromatographic steps (see Figure 1D) and is present in a highly enriched preparation of capping enzyme.

Expression of VITF-A in E.coli

To identify conclusively the viral capping enzyme as VITF-A, we expressed capping enzyme in *E.coli*. The bacterially expressed protein complex has been shown to be functional with respect to the formation of a 5′ cap structure (Guo and Moss, 1990). The genes D1 and D12, encoding the large and small subunit, respectively (Morgan *et al.*, 1984; Niles *et al.*, 1989), were co-expressed using the inducible T7 RNA polymerase system (Studier *et al.*, 1990). Capping enzyme was partially purified from the soluble fraction of the bacterial lysate using Q Sepharose, S Sepharose and phenyl-Superose successively. A control fraction was prepared from a non-transformed strain. Figure 2A shows a Coomassie blue stained SDS−polyacrylamide gel of the phenyl-Superose fractions used. Partially purified capping enzyme obtained from HeLa cells infected with vaccinia virus is included as reference (lane H). Comparing the polypeptide composition of the fraction obtained from bacteria expressing capping enzyme (lane P) with the control fraction (lane M) clearly shows the presence of both subunits of capping enzyme.

Contaminating RNase activity in the phenyl-Superose fractions made it necessary to include heparin as an RNase inhibitor in the reconstituted transcription assay. An initiation complex can be assembled in the presence of ATP which consists of VITF-A, VITF-B, RNA polymerase and DNA. This assembled complex remains able to synthesize intermediate transcripts after challenge with heparin (Vos *et al.*, 1991). Therefore, we preincubated VITF-B, RNA polymerase, DNA and ATP together with fractions containing capping enzyme to allow assembly of a heparin resistant complex. After 10 min at 30°C, heparin and nucleotides were added and productive transcription was started. Using this experimental approach, the *E.coli* produced capping enzyme (Figure 2B, lane P) could functionally substitute for VITF-A (lane H), whereas the control extract could not support intermediate transcription (lane M). Intermediate transcripts were not detected in the presence of only VITF-B and RNA polymerase (lane N). We conclude that VITF-A is the viral capping enzyme.

Intermediate transcription is not terminated in response to the early termination signal

Apart from 5′ cap formation, a second function of capping enzyme is its role in termination of early transcription (Shuman *et al.*, 1987). In order to test whether intermediate transcription also terminates in response to the early termination signal, we used as template a linear fragment containing the I3 tandem early/intermediate promoter upstream of the I3 early termination signal (Schmitt and Stunnenberg, 1988). Correctly initiated and terminated early transcripts are obtained in an early-specific extract derived from virus particles (Figure 3, lane 1). Furthermore, early 'run-off' transcripts are obtained which are not terminated specifically and whose size is determined by the position of truncation of the template. The intermediate-specific reconstituted system consisting of capping enzyme, RNA polymerase and VITF-B only results in intermediate run-

off transcripts (lane 2). Transcripts which are terminated in response to the early termination signal are not obtained.

Capping enzyme is required to form a stable initiation complex

We have determined previously that a committed complex between VITF-B and the DNA can be obtained which is stable to competition with a second template (Vos *et al.*, 1991). A template challenge assay (Fire *et al.*, 1984) was used to determine whether either RNA polymerase or capping enzyme first enters the committed complex (see Figure 4). Two templates, DNA 1 and DNA 2, were used which carry an identical intermediate promoter, but which direct the synthesis of G-less transcripts of different lengths. Both templates were separately preincubated in the presence of VITF-B and ATP to allow the formation of a committed complex. In addition, different combinations of RNA polymerase and capping enzyme were included in a subsaturating amount in the preincubation with DNA 1. A stable complex can potentially be formed between the committed complex and RNA polymerase or capping enzyme during this incubation period. Subsequently, the DNA templates were combined and the missing component(s) for reconstituted transcription were added. Finally, nucleotides were added to allow transcription. If either RNA polymerase or capping enzyme is stably sequestered by the VITF-B–DNA 1 complex during the first preincubation, this would exclude DNA 2 from transcription. Otherwise, factors are redistributed and both templates are transcribed with equal efficiency. Addition of both capping enzyme and RNA polymerase during the first incubation period results in the stable sequestration of RNA polymerase and capping enzyme by the VITF-B–DNA 1 complex, because DNA 1 is preferentially transcribed (Figure 4, lane 4). The low level of transcription of DNA 2 indicates that either a small quantity of RNA polymerase and capping enzyme is not stably sequestered throughout the second incubation or that some reinitiation of transcription occurs. If only capping enzyme (lane 2) or RNA polymerase (lane 3) is added to a VITF-B–DNA 1 complex during the first incubation period, DNAs 1 and 2 become transcribed with the same relative efficiency as when RNA polymerase and capping enzyme are added after combining the two templates (lane 1). The lower level of transcription in lanes 2 and 3 points to an instability of capping enzyme and RNA polymerase during the incubation when they are not sequestered in an initiation complex. Omission of both RNA polymerase and capping enzyme does not result in a detectable level of intermediate transcripts (lane 5). Taken together, these data suggest that a stable complex cannot be formed between a committed VITF-B–DNA complex and either capping enzyme or RNA polymerase alone. However, in the presence of both RNA polymerase and capping enzyme, a stable initiation complex is formed. Formation of a stable initiation complex consisting of VITF-B, DNA, RNA polymerase and capping enzyme is not dependent on ATP, because the same results are obtained when ATP is omitted during the preincubations (data not shown).

Complex formation between capping enzyme and RNA polymerase

Based on the observation that neither RNA polymerase nor capping enzyme can stably interact with the VITF-B–template complex, two possible pathways for the assembly of an initiation complex can be envisioned. A

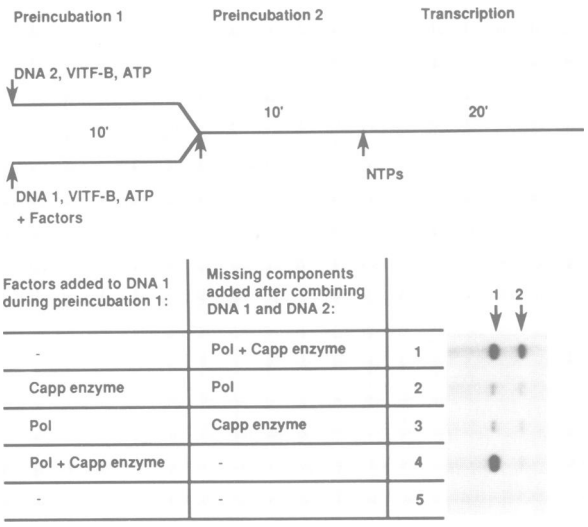

Fig. 4. Sequestration of RNA polymerase and capping enzyme by a VITF-B–DNA complex. A schematic representation of the second template challenge assay is shown. DNA 1 (250 ng of pIΔG[320]) and DNA 2 (250 ng of pIΔG[380]) were separately incubated with 1 μl of VITF-B in the presence of 1 mM ATP in a total volume of 10 μl. To DNA 1 only, a combination of 0.1 μl of RNA polymerase and 0.1 μl of VITF-A was added as indicated. After 10 min at 30°C, the two incubations were combined and the missing components were added with one exception where neither RNA polymerase nor capping enzyme was included (lane 5). After an additional 10 min at 30°C, transcription was started by the addition of all four nucleotides. RNA products were separated on a sequencing gel.

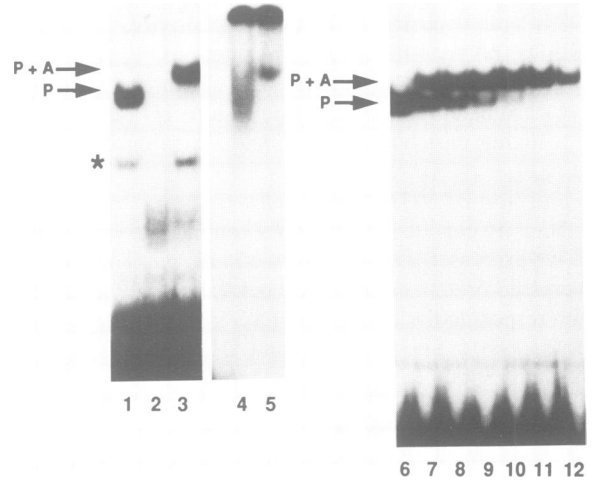

Fig. 5. Bandshift analysis of complex formation between capping enzyme and RNA polymerase. RNA polymerase (lane 1), capping enzyme (lane 2) or both RNA polymerase (0.25 μl) and capping enzyme [1.0 μl, poly(U)–Sepharose fraction] (lane 3) were incubated with a labelled DNA fragment. Labelled GMP–capping enzyme intermediate was used either alone (lane 4) or with RNA polymerase (lane 5) in a binding reaction with an unlabelled DNA fragment. RNA polymerase (0.25 μl) alone or in combination with increasing amounts of capping enzyme (0.0, 0.125, 0.25, 0.5, 1.0, 2.0 or 4.0 μl, lanes 6–12, respectively) were included in the binding reaction. DNA–protein complexes were analysed by native 5% PAGE. The positions of both the RNA polymerase–DNA complex and the RNA polymerase–capping enzyme–DNA complex are indicated by arrows marked P and P + A, respectively.

ternary complex is transiently formed between the committed complex and either RNA polymerase or capping enzyme, whereby stable complex formation relies on the subsequent entry of the missing component. Alternatively, RNA polymerase and capping enzyme might first form a complex in solution before entry into the committed complex. We therefore investigated whether a complex between RNA polymerase and capping enzyme could be detected using a gel retardation assay.

Purified RNA polymerase is able to bind DNA and to retard a labelled DNA probe in a native polyacrylamide gel (Figure 5, lane 1, complex marked P). This binding is not intermediate promoter sequence dependent and can be obtained with an unrelated double-stranded or single-stranded oligonucleotide. Direct evidence for the presence of RNA polymerase in the retarded complex was obtained by formation of a ternary complex between unlabelled DNA, RNA polymerase and labelled RNA, which is synthesized during the binding reaction. This complex was sensitive to RNase A digestion and migrated with a similar mobility to the binary complex (data not shown). A contaminating protein in the RNA polymerase fraction results in a retarded complex with a higher mobility (indicated with an asterisk). Addition of partially purified capping enzyme to the binding reaction with RNA polymerase results in a shift with a lower mobility which we interpret as a complex between RNA polymerase and capping enzyme (lane 3). By itself, capping enzyme does not bind the probe (lane 2). Increasing the amount of capping enzyme shows a gradual shift of the polymerase complex into a higher order complex (lanes 6–12). Finally, we performed bandshift experiments using an [α-^{32}P]GTP-labelled enzyme–guanylate complex together with a non-radiolabelled DNA probe to confirm the presence of capping enzyme within the low mobility complex. Capping enzyme gives rise to a non-discrete migration of protein into the gel in the absence of RNA polymerase (lane 4). Addition of RNA polymerase (lane 5) results in a discrete retarded complex with a mobility comparable to that seen in lane 3. We conclude that capping enzyme can form a stable complex with RNA polymerase.

Discussion

It has been shown that the virally encoded capping enzyme is essential for virus propagation (Niles *et al.*, 1989). To date, three different functions can be attributed to capping enzyme. The first one is the formation of an m^{7}G(5′)pppN cap structure (Martin *et al.*, 1975). Furthermore, capping enzyme functions as a cofactor in termination of early transcription (Shuman *et al.*, 1987). Here, we report on the role of the enzyme in the initiation of intermediate transcription.

We have previously defined three activities which are required to reconstitute transcription of intermediate genes *in vitro* (Vos *et al.*, 1991). We demonstrate that one of these activities, originally called VITF-A, is the viral capping enzyme which is expressed in the early phase of infection (Morgan *et al.*, 1984). The protein has been purified extensively from HeLa cells infected with vaccinia virus. Direct evidence for its role in intermediate transcription was obtained by expression of capping enzyme in *E.coli* and its ability to substitute VITF-A in a reconstituted transcription system.

The function of capping enzyme appears to be dependent on the promoter context. In early transcription, capping enzyme is not required for transcription initiation and elongation, whereas there is a requirement for capping enzyme at the level of transcription termination (Shuman *et al.*, 1988). In contrast, intermediate transcription initiation is dependent upon the presence of capping enzyme. Termination of transcription in response to an early termination signal was not observed, despite the requirement for capping enzyme in intermediate transcription. This suggests that initiation complexes can be formed which are either termination competent or incompetent. The ability of RNA polymerase to terminate might therefore originate from additional proteins associated with the elongating polymerase complex. One possibility is that the early transcription factor, VETF, remains associated with the transcribing polymerase and in combination with capping enzyme renders it termination sensitive. Alternatively, the ability to terminate relies on a modification of the RNA polymerase. A parallel can be drawn with certain cellular U snRNA genes transcribed by RNA polymerase II, where part of the termination signal is contained within the promoter. Transcription complexes are assembled on these promoters which are competent to recognize specific termination signals associated with this class of genes (Hernandez and Weiner, 1986; Neuman de Vegvar *et al.*, 1986).

With regard to intermediate-specific transcription initiation, VITF-B has been defined as a stage-specific transcription factor. VITF-B confers intermediate promoter specificity on an early-specific extract prepared from virus particles which contains both viral RNA polymerase and capping enzyme. The first step in transcription initiation is the binding of VITF-B to the promoter. A complex is formed which is stable to challenge with a second template (Vos *et al.*, 1991). The VITF-B–DNA complex can stably sequester both RNA polymerase and capping enzyme to form a complex which is stable to challenge with a second VITF-B–DNA complex. Interestingly, neither RNA polymerase nor capping enzyme alone is able to assemble into a stable complex with the VITF-B–DNA complex. The observation that capping enzyme can interact with RNA polymerase in the absence of VITF-B, as shown in gel retardation assays, indicates that RNA polymerase first forms a complex with capping enzyme before entry into the VITF-B–DNA complex. This RNA polymerase–capping enzyme complex can be obtained using either a double- or single-stranded oligonucleotide indicating that DNA binding is not a prerequisite for complex formation. Furthermore, capping enzyme does in part co-elute with RNA polymerase during ionic exchange and/or sieving column chromatography (J.-C.Vos and H.G.Stennenberg, unpublished observation). Thus, capping enzyme is required in a step prior to transcription initiation. It will be of interest to determine whether capping enzyme can contribute to the stability of an early transcription initiation complex. The function of capping enzyme in vaccinia virus intermediate transcription is reminiscent of the role of TIF-1A in mouse RNA polymerase I transcription (Schnapp *et al.*, 1990). TIF-1A binds to RNA polymerase I and converts it into an active form with respect to specific transcription of rDNA. Likewise, a growth-regulated modification of *Acanthamoeba* RNA polymerase I determines whether or not RNA polymerase I can form a complex with a transcription initiation factor (Bateman and Paule, 1986).

In HeLa cell extracts, efficient capping of RNA polymerase II transcripts has been reported (Coppola *et al.*, 1983; Samuels *et al.*, 1984). However, formation of a cap structure is not required for RNA polymerase II transcription and nor does it enhance transcription (Bunick *et al.*, 1982; Samuels *et al.*, 1984). These results, together with the observation that most of the cellular capping enzyme is recovered in fractions which are not required for accurate transcription (Samuels *et al.*, 1982), do not point to a structural role of the cellular capping enzyme in RNA polymerase II transcription. We have not yet been able to demonstrate whether vaccinia virus capping enzyme has an essential enzymatic role in addition to its structural one in intermediate transcription. One possible way of excluding cap synthesis is to perform transcription in the absence of any GTP. However, addition of ultrapure nucleotides (ATP, CTP and UTP) to the transcription reaction nevertheless results in a concentration of GTP which is sufficiently high to load capping enzyme with a GMP residue. Alternatively, the incorporation of a non-cleavable γ-phosphate at the 5′ end of the transcript would make the RNA refractory to capping. In the presence of AMP-PNP a very low, but reproducible level of transcription can be obtained (Vos *et al.*, 1991). This residual level of transcription could reflect either capping enzyme independent transcription or an inefficient usage of AMP-PNP as starting nucleotide. It remains an attractive option that capping of the nascent transcript is an essential step in productive intermediate transcription.

Materials and methods

Protein purification

VITF-A was purified from a cytoplasmic extract prepared from HeLa cells infected with vaccinia virus in the presence of hydroxyurea. Chromatography of VITF-A on DEAE−Sephacel, heparin−Sepharose and poly(U)−Sepharose was as described (Vos *et al.*, 1991). VITF-A derived from the poly(U)−Sepharose column was applied to an FPLC Mono S column at 100 mM NaCl in S buffer and eluted with a gradient from 100 to 500 mM NaCl. VITF-A eluted at 175 mM NaCl. One volume of 1 M $(NH_4)_2SO_4$ in S buffer was added and the protein loaded on an FPLC phenyl-Superose (Pharmacia) column. The column was developed with a gradient from 500 to 0 mM $(NH_4)_2SO_4$ and VITF-A eluted at 100 mM $(NH_4)_2SO_4$. Column fractions were dialysed to 100 mM NaCl in S buffer (20 mM HEPES pH 7.9, 15% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT). RNA polymerase and VITF-B were purified as described previously (Vos *et al.*, 1991).

Transcription conditions and templates

Standard transcription assays and the plasmids pIΔG[320] and pIΔG[380] were as described (Vos *et al.*, 1991). Plasmid pI3TS is a pUC19 derivative, in which a *PstI*−*XbaI* fragment of the vaccinia virus genome containing the I3 promoter is inserted into the corresponding sites in the polylinker. Subsequently, an *HpaII*−*DraI* viral genomic fragment containing the I3 termination signal was inserted in the *SmaI* site with the TTTTTAT sequence in the non-transcribed strand with regard to the I3 promoter (Schmitt and Stunnenberg, 1988). pI3TS was digested with *PvuII* and the 1.1 kb fragment was gel purified. Transcription was performed with 100 ng of template using standard conditions with 0.5 mM GTP and in the absence of 0.1 mM 3′-OMeGTP.

Preparation of E.coli extracts

Overnight cultures of *E.coli* BL21(DE3)pLysE (Studier *et al.*, 1990) containing plasmid pETD12D1 (Guo and Moss, 1990) were used to inoculate 2 litres of L broth. After the bacteria reached an $OD_{595\ nm}$ of 0.2, T7 RNA polymerase was induced by addition of 0.1 mM IPTG; bacteria were collected by centrifugation after 2.5 h. Bacteria were resuspended in S buffer containing 100 mM NaCl and lysed by one cycle of freeze−thawing. After centrifugation for 30 min at 15 000 r.p.m., the supernatant was passed through Q Sepharose FF. The flowthrough was applied to an S Sepharose FF column equilibrated at 100 mM NaCl in S buffer. Capping enzyme was recovered by step elution using 250 mM NaCl in S buffer. After dialysis against 2 M KCl in S buffer, proteins were applied on an FPLC phenyl-Superose column which was developed with a gradient from 2 M to 50 mM KCl. The peak fraction of capping enzyme was eluting at 1.2 M KCl and was dialysed against S buffer containing 100 mM KCl.

Gel retardation

Conditions for binding: 15 mM HEPES (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 7.5% glycerol, 0.25 mM DTT, 0.04% NP40, 1 μg BSA, 2 μg poly(d[I-C]) in 20 μl with 10 fmol of a 60 bp DNA fragment. Binding was for 30 min on ice. Complexes were separated on a 5% polyacrylamide (29:1) gel in 0.5× TBE which was run at 4°C. The double-stranded oligonucleotide consisted of I3 intermediate promoter sequences (−33 to +9 with respect to the site of transcription initiation) preceded and followed by GGGGCCC.

Transguanylylation

Capping enzyme was incubated with 5 μCi of [α-^{32}P]GTP in 25 μl for 15 min at 30°C in 50 mM Tris pH 8.1, 4 mM $MgCl_2$, 4 mM DTT (Shuman and Hurwitz, 1981) and TCA precipitated using 5 μg of BSA as carrier. The pellet was washed with 50% acetone, dried, resuspended in Laemmli buffer and applied to SDS−PAGE.

Labelled capping enzyme to be used in the gel retardation assay was passed over a G50 gel filtration spin column equilibrated with S buffer containing 100 mM NaCl to remove free [α-^{32}P]GTP.

Acknowledgements

We would like to thank Dr B.Moss (NIH, Bethesda) for generously providing us with plasmid pETD12D1. Furthermore, we are grateful to our colleagues for helpful discussions and assistence and Iain Mattaj for critically reading the manuscript.

References

Bateman,E. and Paule,M.R. (1986) *Cell*, **47**, 445−450.
Broyles,S.S., Yuen,L., Shuman,S. and Moss,B. (1988). *J. Biol. Chem.*, **263**, 10754−10760.
Bunick,D., Zandomeni,R., Ackerman,S. and Weinmann,R. (1982) *Cell*, **29**, 877−886.
Coppola,J.A., Field,A.S. and Luse,D.S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1251−1255.
Fire,A., Samuels,M. and Sharp,P.A. (1984) *J. Biol. Chem.*, **259**, 2509−2516.
Guo,P. and Moss,B. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4023−4027.
Hernandez,N. and Weiner,A.M. (1986) *Cell*, **47**, 249−258.
Martin,S.A., Paoletti,E. and Moss,B.(1975) *J. Biol. Chem.*, **250**, 9322−9329.
Morgan,J.R., Cohen,L.K. and Roberts,B.E.(1984) *J. Virol.*, **52**, 206−214.
Moss,B. (1990) *Annu. Rev. Biochem.*, **59**, 661−688.
Neuman de Vegvar,H.E., Lund,E. and Dahlberg,J.E. (1986) *Cell*, **47**, 259−266.
Niles,E.G., Lee-Chen,G.-J., Shuman,S., Moss,B. and Broyles,S.S. (1989) *Virology*, **153**, 513−522.
Samuels,M., Fire,A. and Sharp,P.A. (1982) *J. Biol. Chem.*, **257**, 14419−14427.
Samuels,M., Fire,A. and Sharp,P.A. (1984) *J. Biol. Chem.*, **259**, 2517−2525.
Sawadogo,M. and Roeder,R.G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4394−4398.
Schmitt,J.F.C. and Stunnenberg,H.G. (1988) *J. Virol.*, **62**, 1889−1897.
Schnapp,A., Pfleiderer,C., Rosenbauer,H. and Grummt,I. (1990) *EMBO J.*, **9**, 2857−2863.
Shuman,S and Hurwitz,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 187−191.
Shuman,S., Broyles,S.S. and Moss,B. (1987) *J. Biol. Chem.*, **262**, 12372−12380.
Shuman,S. and Moss,B. (1989) *J. Biol. Chem.*, **263**, 6220−6225.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorf,J.W. (1990) *Methods Enzymol.*, **185**, 60−89.
Vos,J.C. and Stunnenberg,H.G. (1988) *EMBO J.*, **7**, 3487−3492.
Vos,J.C., Sasker,M. and Stunnenberg,H.G. (1991) *Cell*, **65**, 105−113.

Received on April 29, 1991; revised on May 29, 1991