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Vaccinia virus early gene transcription termination factors VTF and Rap94 interact with the U9 termination motif in the nascent RNA in a transcription ternary complex

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ABSTRACT

The vaccinia virus core contains a 195 kb double stranded DNA genome, a multi-subunit RNA polymerase, transcription initiation and termination factors and mRNA processing enzymes. Upon infection, vaccinia virus early gene transcription takes place in the virus core. Transcription initiates at early promoters and terminates in response to a termination motif, UUUUUNU, in the nascent mRNA. Early gene transcription termination requires the vaccinia virus termination factor, VTF, a single stranded DNA-dependent ATPase, and NPH I, the Rap94 subunit of the virion RNA polymerase, as well as the presence of the UUUUUNU motif in the nascent RNA. The position of UUUUUNU in the ternary complex suggests that it serves as a site of interaction with one or more components of the transcription termination complex. In order to identify the factor(s) that interact with UUUUUNU a series of direct UV photo crosslinking and ribonuclease A protection studies were undertaken. Through these analyses both VTF and Rap94 were shown to interact with UUUUUNU in the isolated ternary complex. Evidence indicates that the interaction is not mutually exclusive. VTF was shown to bind to UUUUUNU through the N-terminal domain of the large D1 subunit. Furthermore, VTF protects from RNAse A digestion both the 5′ region of the nascent transcript as well as a large central component containing UUUUUNU. The addition of an oligonucleotide containing the ^{5Br}U9 sequence both directly inhibits transcription termination, *in vitro* and inhibits UV photo crosslinking of VTF to the nascent RNA in the ternary complex. These results support a model in which the availability of the UUUUUNU motif outside of the transcribing RNA polymerase permits binding of both transcription termination factors, VTF and Rap94, to UUUUUNU. The assembly of this termination complex initiates the transcription termination sequence.

Introduction

Poxviruses comprise a highly successful group that infects hosts as diverse as insects and primates (Moss, 2007). Poxviruses are large complex viruses that contain a nucleoprotein core wrapped in one or two phospholipid layers. The core harbors a single double stranded genome that contains from 150 to 300 genes depending upon the specific virus. In addition, the core possesses a multi-subunit RNA polymerase and associated transcription initiation and termination factors, in addition to mRNA processing enzymes. Poxviruses are unique among DNA viruses in that they carry on their replication cycle in the cytoplasm of infected cells. Like other large DNA viruses, genes are transcribed in three temporal classes that differ in their associated transcription factors and in the template location and conformation (Broyles, 2003). Early genes are transcribed within the infecting virus core utilizing the core-associated RNA polymerase and transcription factors. The core actually contains two multi-subunit RNA polymerases (Ahn and Moss, 1992; Kane and Shuman, 1992). One form possesses eight virus encoded subunits and exhibits the general architecture of a eukaryotic nuclear RNA polymerase. The other form, which is active in early gene transcription, possesses an additional subunit, Rap94. Evidence indicates that this latter RNA polymerase is active only on early genes (Ahn et al., 1994; Deng and Shuman, 1994; Wright and Coroneos, 1995; Condit et al., 1996) and that newly synthesized RNA polymerase transcribes genes belonging to the latter two classes (Hooda-Dhingra et al., 1989).

Early gene transcription terminates in a factor and sequence dependent manner (Rohrmann et al., 1986; Yuen and Moss, 1986). Termination requires the vaccinia termination factor, VTF (Shuman et al., 1987), which is a multi-functional heterodimer that also serves as the viral mRNA capping enzyme (Ensinger et al., 1975; Venkatesan et al., 1980) and an intermediate gene transcription initiation factor (Vos et al., 1991). In addition, termination needs energy provided by ATP hydrolysis (Shuman et al., 1987; Hagler et al., 1994) catalyzed by a virus encoded enzyme, nucleoside triphosphate hydrolyase I, NPH I (Deng and Shuman, 1996; Deng and Shuman, 1998; Christen et al., 1998). NPH I possess motifs present in superfamily II helicases (Christen et al., 1998) but helicase

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activity has not been demonstrated, to date. The C-terminus of NPH I binds to the N-terminus of the Rap94 subunit of the virion RNA polymerase (Mohamed and Niles, 2000). All evidence indicate that this interaction is essential for termination, *in vitro* (Mohamed and Niles, 2000, 2001; Piacente et al., 2003). Finally, the conserved sequence UUUUUNU, which resides in the nascent RNA at a position 30 to 50 bases from the poly A addition site is essential for transcription termination (Yuen and Moss, 1986, 1987; Shuman and Moss, 1988). Prior results indicate that the UUUUUNU termination motif interacts with one or more termination factors to initiate the termination pathway (Hagler et al., 1994).

Key to understanding the termination pathway is the identification of the UUUUUNU recognition protein(s) since it is likely that the binding to this motif is an early step in the termination sequence. Likely candidates include VTF, which is known to be an RNA binding protein (Luo and Shuman, 1993; Higman et al., 1994; Myette and Niles, 1996b). Prior experiments failed to demonstrate sequence specificity, although VTF did show a preference for oligo U (Luo and Shuman, 1993). Since VTF catalyzes the first three steps of mRNA cap formation, which requires binding to the 5′ end of nascent mRNA, sequence specificity for RNA binding would not be anticipated. NPH I must bind ssDNA in order to exhibit ATPase activity (Paoletti and Moss, 1974; Christen et al., 1999). Although ssRNA does not stimulate ATPase activity, RNA competes for binding with ssDNA and acts as an inhibitor of NPH I. Again, no sequence specificity was observed for ssRNA inhibition of NPH I activity. Alternatively, any of the 9 subunits of the virion RNA polymerase may harbor the UUUUUNU recognition site. Finally, an as yet unidentified protein may serve as the UUUUUNU binding partner. This protein could act in *trans* as a dissociable factor. Alternatively, it may act in *cis* as a component of the RNA, DNA, RNA polymerase ternary complex.

In this report, the results of direct UV photo crosslinking and ribonuclease A protection studies are presented, which show that in an isolated ternary complex multiple RNA polymerase subunits can interact with the U9 termination motif in the nascent RNA. Importantly, the known termination factors, VTF and Rap94 each bind the termination motif. RNAse A sensitivity studies demonstrate that VTF can protect up to 50 bases of the exposed transcript through interactions both with the 5′ end region and with the U9 motif in the central component of the RNA. Competition studies show that an oligonucleotide containing ^{5Br}U9 both inhibits transcription termination and crosslinking of U9 to VTF supporting a model in which VTF binding to the U9 termination motif is a required step in early gene transcription termination.

Results

Construction of a termination competent ternary complex

Deng et al. (1996) described the construction of a magnetic bead-bound linear double stranded DNA template, Ter29 that they employed in vaccinia virus early gene transcription studies, Fig. 1A. This template possesses a strong early promoter that initially directs transcription through a short 20 base pair G-less cassette in the absence of GTP and the presence of 3′ *O* methyl GTP. Step 1 transcription yields a stable ternary complex that contains a 21 nucleotide transcript, G21, with 3′ *O* methyl

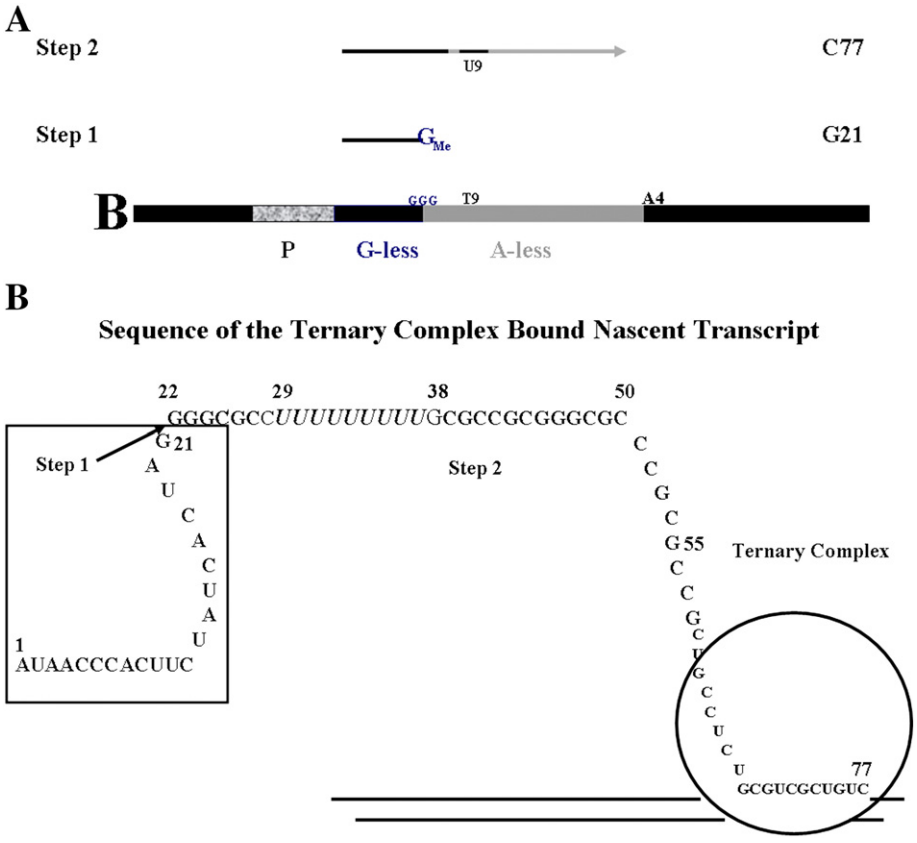

Fig. 1. A. Map of the Ter29 kas template. The Ter29 template was described by Deng et al. (1996). Ter29 is a linear double stranded DNA that is linked to strepavidin beads (B). There is a strong early promoter (P) that drives transcription through a 20 base pair G-less cassette in Step 1 transcription, yielding a 3′ *O* methyl G21 RNA. Beads can be collected, washed and elongated in Step 2 in the absence of ATP to yield a 77 base transcript, C77, placing the ternary complex at the A4 sequence that flanks the A-less cassette. B. This cartoon presents the sequence of the entire C77 transcript bound in a ternary complex. RNA is numbered from the first base in the transcript. The Step 1 sequence is boxed. The rest of the sequence can be radiolabeled in Step 2. If α^{32}P UTP is employed the radioactivity is restricted to the U9 termination motif and to the region between U60 and U76 that resides within the RNA product channel of the RNA polymerase. The circle represents RNA polymerase present in the ternary complex.

GMP at the 3′ terminus. Adjacent to the G-less sequence is a 57 base pair A-less cassette that harbors a T9 stretch in the non-template strand, beginning at nucleotide 29, where nucleotide 1 is the first base of the transcript. After washing the beads, a second transcription reaction, Step 2, can be conducted in the absence of ATP, which moves the ternary complex to a position at the end of the A-less cassette. The Step 2 ternary complex possesses a 77 base nascent transcript, Fig. 1B, which has the U9 termination motif located outside of the RNA polymerase product channel. The 3′ end of the U9 motif is 41 bases from the 3′ end of the nascent transcript in a location similar to that observed in poly A^+ transcripts, *in vivo*. We modified the sequence of Ter29 kas so that certain T bases in the non-template strand were replaced with C or G, in the region between positions 41 and 57, Fig. 1B. This allowed retention of Ts in the non-template strand both in the Step 1 sequence and in the region between base pairs 60 and 76. This permitted us to selectively label portions of the transcript with α^{32}P UTP between U2 and U19 in Step 1 transcription. Importantly, it would allow us to label the U9 termination motif between U29 and U37, and also U60 to U76 in Step 2 transcription. Selective labeling would permit us to evaluate interactions in the 5′ region of the transcript by incorporating radioactivity during Step 1 transcription. Furthermore, it would permit us to focus on interactions between the U9 termination motif and its recognition factor through incorporating α^{32}P UTP in Step 2 transcription. Finally, the 3′ terminal UMP residues between U60 and U76 would be expected to reside in the RNA product channel of the RNA polymerase since they lie in a region of the nascent transcript that is protected from RNAse A digestion (Hagler and Shuman, 1992a). This latter segment would serve as an internal control for evaluating protein/RNA interactions.

Direct UV photo crosslinking of proteins in the ternary complex to the nascent RNA

UV photo crosslinking affords an opportunity to identify proteins that are in close contact with radiolabeled RNA. A positive result provides convincing evidence that a protein binds to the RNA. However, a negative result does not necessarily demonstrate the lack of contact. This is due to the fact that the UV photo activated base must lie both close to and in a favorable position to form a covalent bond with an amino acid side chain. In addition, since crosslinking is an irreversible event, the extent of crosslinking cannot be used to measure affinity. With these caveats in mind, we employed this approach to attempt to identify proteins in a transcription ternary complex that lie in close contact with the U9 termination motif. α^{32}P UTP was incorporated into the nascent RNA in the bead-bound ternary complex either in the first or second transcription reaction. Thus, UMP located either between positions 2 and 19, or between positions 29 and 76, was selectively radiolabeled, permitting evaluation of protein/RNA interactions either in the 5′ region or the central region of the nascent transcript. As an initial test, ternary complexes were prepared after Step 1 transcription, which contained a short, radiolabeled, G21 base transcript. After washing, the bead-bound templates were treated with UV light in the presence or absence of added VTF. Ternary complexes were treated with DNAse I and RNAse T1, denatured and separated by gel electrophoresis and visualized by autoradiography. In Fig. 2A, a single prominent radioactive component was observed in the absence of added VTF. When VTF was added prior to irradiation, a minor faster migrating product is also seen. Immunoprecipitation with polyclonal antibodies directed against the large subunits of the viral RNA polymerase and the large, D1 subunit of VTF confirmed the identity of the radioactive components (data not shown). The major protein crosslinked to the nascent Step 1 RNA is the 146 kDa subunit of the viral RNA polymerase, which identifies the location of the product RNA channel in this subunit.

Bead-bound ternary complexes were prepared which were radiolabeled with α^{32}PUTP during Step 2 transcription. Radioactive UMP was located both in the U9 termination motif positioned outside of the RNA polymerase, and also in the 3′ region of the transcript residing in the RNA product channel. UV photo crosslinking was carried out in the absence or the presence of added VTF and after nuclease digestion and denaturation of the proteins, the radiolabeled components were separated by gel electrophoresis and the migration positions identified by autoradiography, Fig. 2B. In the absence of added VTF, three prominent components can be identified. The upper band corresponds to the migration position of the two large subunits of the virion RNA polymerase. The second component corresponds to the mobility of the large subunit of VTF. It will be demonstrated that this band contains both Rap94 and VTF-D1. VTF is a heterodimer that contains two subunits: VTF-D1 (97 kDa) and VTF-D12 (32 kDa). A small crosslinking product of about 18 kDa molecular weight is also present. When VTF is added prior to photo crosslinking additional bands can be seen. The large RNA polymerase subunits are present as well as an enhanced level of VTF

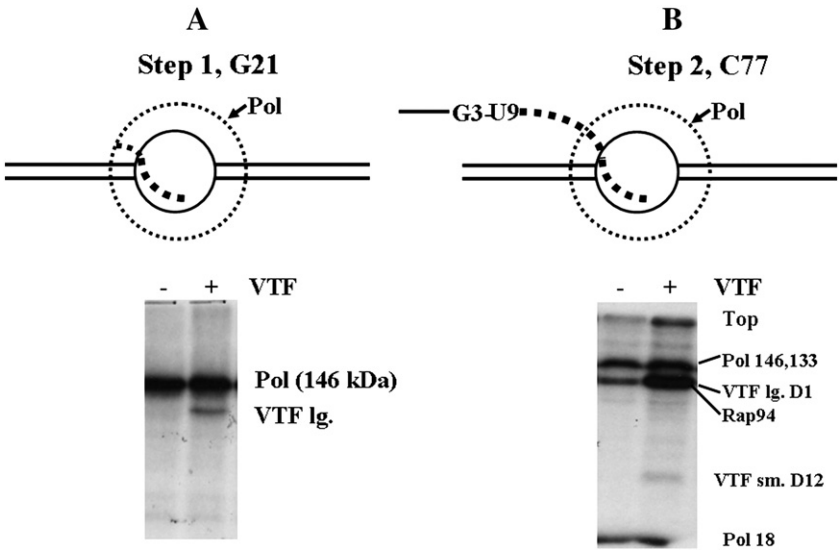

Fig. 2. UV photo crosslinking of proteins present in the ternary complex at the end of Step 1 transcription (A) or Step 2 transcription radiolabeled in Step 2 (B). A. The Step 1 transcript radiolabeled with α^{32}PUTP is denoted by the dashed line. B. The Step 2 transcript contains two regions. The 5′ 28 bases lack radioactivity. From position 29 to 76, U residues are radiolabeled. This region is denoted by a dashed line. For both A and B, the dashed circle represents RNA polymerase in the ternary complex. Crosslinking was carried out in the presence or absence of 2 pmol VTF. The radiolabeled products were separated by gel electrophoresis and observed by autoradiography. The radiolabeled protein components were identified by immunoprecipitation with polyclonal antibodies.

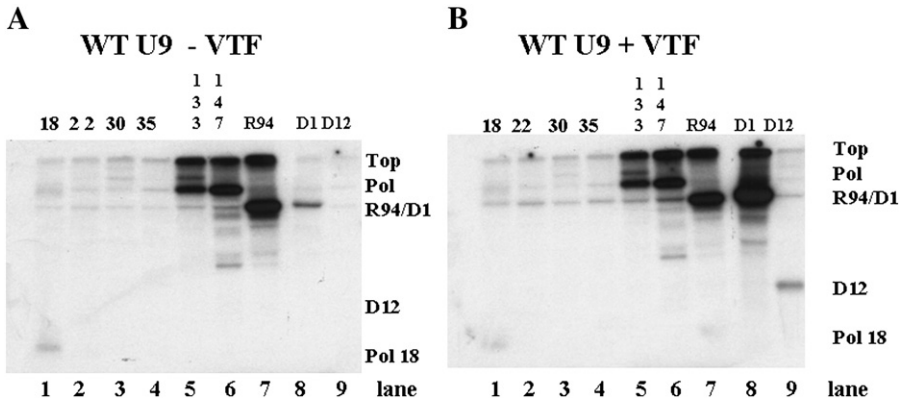

Fig. 3. Immunoprecipitation of UV photo crosslinked proteins. Ternary complexes were prepared with the nascent C77 RNA radiolabeled in Step 2 with α^{32}PUTP. Complexes were UV photo crosslinked in the absence or presence of 2 pmol of VTF, nuclease treated and denatured with SDS. Proteins were immunoprecipitated with polyclonal antibodies raised against individual subunits of the vaccinia virus RNA polymerase or mRNA capping enzyme (VTF) and observed by autoradiography subsequent to gel electrophoresis. The numbers at the top of each lane refer to the proteins to which antibodies were raised, e.g. 18 refers to Rpo18, a RNA polymerase subunit. On the right side of each panel, the precipitated bands are identified. Pol, Rpo 146 and Rpo 133; R94, Rap94; D1 and D14, VTF subunits; Pol 18, Rpo18. Variable low levels of crosslinking were observed to both the 30 kDa subunit of RNA polymerase and to NPH I.

large subunit. Additional minor lower molecular components are also observed. A crosslinking product of about 32 kDa is present as well as the 18 kDa component seen in the absence of VTF. Washing the ternary complexes with up to 1 M potassium acetate did not alter the amount or distribution of the crosslinking products (data not shown).

In order to identify the proteins capable of being crosslinked to the nascent RNA radiolabeled in Step 2 transcription, a series of immunoprecipitations were conducted. Ternary complexes were constructed and UV photo crosslinking was carried out in the absence or presence of added VTF. After nuclease treatment, protein complexes were disrupted with a low level of detergent prior to antibody addition. In Figs. 3A,B the results are presented. The numbers on top of each lane identifies the RNA polymerase subunit specific antiserum employed. In the absence of added VTF, the largest component resolved by gel electrophoresis in Fig. 2B actually contains the 133 kDa and 147 kDa subunits of the RNA polymerase. In addition, the small 18 kDa product is also identified as a RNA polymerase subunit. On occasion, a variable level of crosslinking to a 30 kDa RNA polymerase subunit and to NPH I can be seen, as well. In the absence of added VTF, Fig. 3A, a low level of radioactive D1 is observed. VTF is effectively washed from the ternary complex after Step 1 transcription but a low and variable level remains. However, in Fig. 2B, a crosslinked protein was observed that comigrates with the D1 subunit of VTF. Results in Fig. 3A demonstrate that the comigrating radiolabeled component is the Rap94 subunit of the viral RNA polymerase. When VTF is added prior to treatment with UV light, one now observes a substantial level of crosslinking to both the large D1 and small D12 subunits of VTF. Hagler et al. (1994) also provided evidence for an interaction between VTF and the central region of the nascent transcript. Thus, the U9 termination motif in the nascent transcript can be photo crosslinked to multiple subunits in the RNA polymerase, and also to two components of the transcription termination system VTF and Rap94. Based on their known function in early gene transcription termination, further studies focused on both VTF and Rap94. It remains to be seen if the observed minor interactions seen with the 18 kDa, 133 kDa and 146 kDa RNA polymerase subunits have a functional significance in terms of early gene transcription termination.

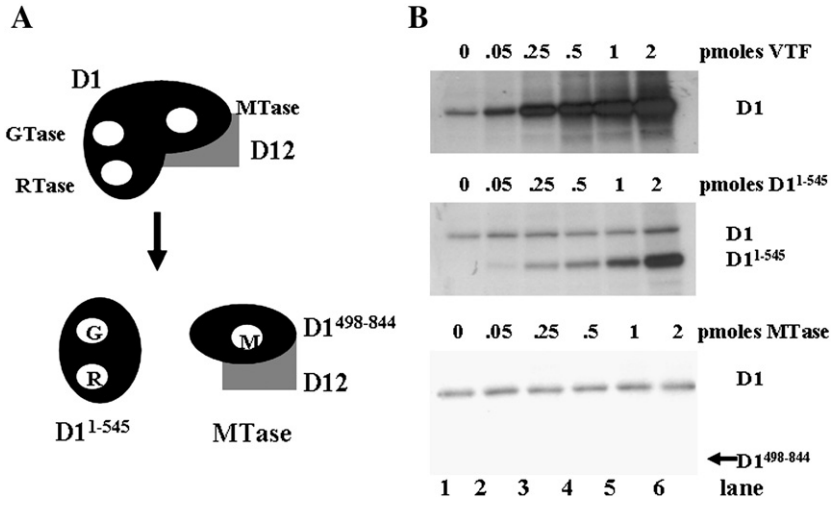

Fig. 4. The U5NU termination motif UV photo crosslinks to the N-terminal domain of the VTF D1 subunit. A. A cartoon is presented that depicts the heterodimeric structure of VTF, containing the 97 kDa D1 subunit and the 32 kDa D12 subunit. The three catalytic sites of the mRNA capping enzyme can be separated into two active domains. The mRNA triphosphatase (R) and the guanylyltransferase (G) active sites reside in the N-terminal 60 kDa domain containing amino acids 1 to 545. The N-7-methyltransferase active site (M) is in the C-terminal region of D1 between amino acids 498 and 844 and requires association of the D12 subunit for full catalytic activity. B. Ternary complexes were prepared with nascent RNA labeled in Step 2 with α^{32}PUTP. Complexes were incubated with variable levels of VTF, with $D1^{1-545}$ or the MTase domain, $D1^{498-844}$D12, and treated with UV light. After nuclease treatment, and denaturation, radiolabeled proteins were analyzed by immunoprecipitation with antibodies directed against a fragment of the VTF D1 protein, amino acids 147 to 621. In the case of the MTase domain, the level of crosslinking to either $D1^{498-844}$ or the D12 subunit was below the level of detection.

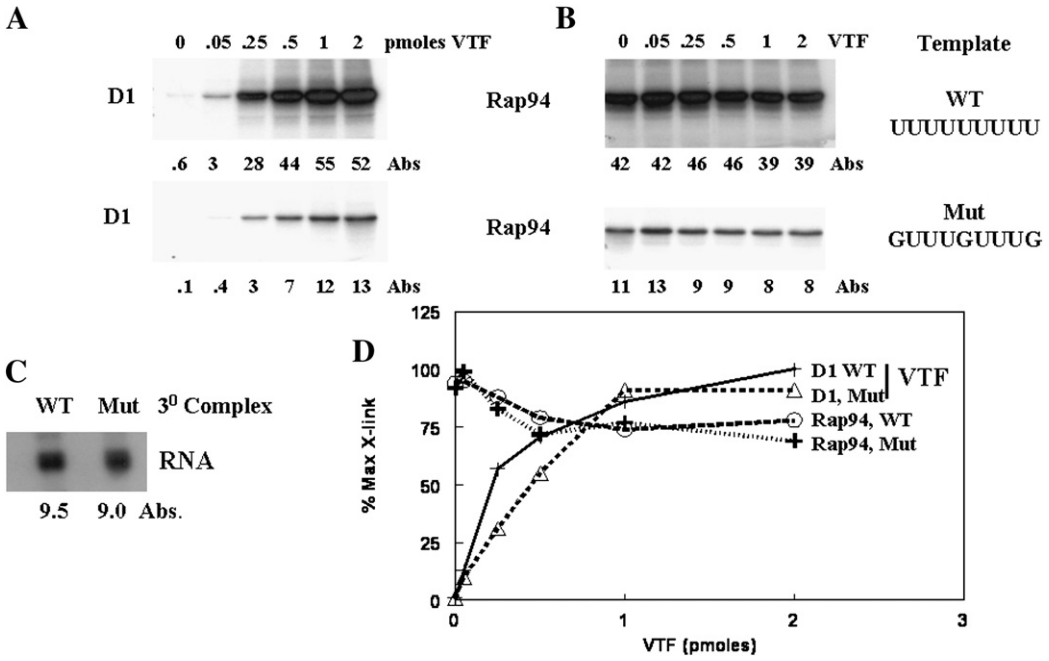

Fig. 5. Crosslinking of Rap94 and VTF to the U5NU motif is not mutually dependent. Ternary complexes were prepared using a template that possessed a wild type or a mutant termination sequence. Step 2 transcription of each template yielded ternary complexes that had nascent transcripts containing either a wild type or a mutant termination motif. UV photo crosslinking was conducted in the absence or presence of increasing amounts of VTF. The radiolabeled ternary complexes were nuclease treated, denatured and the radiolabeled D1 and Rap94 proteins were collected by immunoprecipitation. After gel electrophoresis precipitated proteins were observed by autoradiography and the intensities of the bands were determined by densitometry. A. Antibodies against D1 were employed. B. Anti-Rap94 was used. C. Transcripts synthesized on the wild type or mutant templates were quantified by densitometry. D. This is a graphical representation of the levels of immunoprecipitated crosslinked D1 and Rap94. Each point is the average of three independent determinations. (+) WT U9 and D1; (Δ) Mut U9 and D1; (o) WT U9 and Rap94; and (+) Mut U9 and Rap94. Abs refers to the densitometry of each component determined by densitometry of the exposed films.

The N-terminal domain of the large D1 subunit of VTF crosslinks to the U9 motif

VTF also catalyzes the first three steps in the formation of the mRNA cap 0 structure (Ensinger et al., 1975; Venkatesan et al., 1980). Prior studies demonstrated that the mRNA triphosphatase and guanylyltransferase active sites reside in the N-terminal 60 kDa domain of the large D1 subunit (Shuman and Morham, 1990; Higman et al., 1992). This domain can either be released by protease cleavage of the D1/D12 heterodimer or can be expressed in active form in *Escherichia coli* as $D1^{1-545}$ (Myette and Niles, 1996a). The N7 guanine methyltransferase active site is present in a heterodimer that contains $D1^{498-844}$ and the D12 subunit, Fig. 4A (Higman et al., 1992, 1994). In order to identify the region of VTF that interacts with the U9 motif in the transcription ternary complex varying concentrations of VTF or either active domain of VTF were incubated with isolated Step 2 ternary complexes, treated with UV light, denatured and immunoprecipitated with antibodies directed against the N- or C-terminal region of D1. The precipitated products were separated by gel electrophoresis and observed by autoradiography. In the top panel of Fig. 4B a dose dependent variation in crosslinking of full length D1 is apparent. When the N-terminal domain, $D1^{1-545}$, was evaluated two components were observed, Fig. 4B, middle panel. The top band is the endogenous D1 subunit remaining in the washed ternary complex. Below is the dose dependent crosslinking of U9 to $D1^{1-545}$, demonstrating that VTF is able to interact with U9 through the N-terminal domain. When varying concentrations of the methyltransferase domain were evaluated, only crosslinking to the endogenous VTF D1 subunit is observed. Thus, in the isolated ternary complex, VTF interacts with the U9 termination motif through a region present in the N-terminal domain of the large subunit.

Interaction of VTF and Rap94 to the U9 termination motif is not mutually exclusive

Immunoprecipitation analysis demonstrates that both the Rap94 subunit of the virion RNA polymerase and VTF can be crosslinked to the U9 termination motif in the ternary complex. In order to determine whether the interaction of U9 with one component impacts on crosslinking to the other component, UV photo crosslinking was conducted with Step 2 labeled ternary complexes at different levels of added VTF. Subsequent to incubation of the ternary complexes with VTF, samples were irradiated with UV light, nuclease treated, denatured and immunoprecipitated with antibodies directed against Rap94 and the D1 subunit of VTF. In Figs. 5A,B, top panels, the extent of crosslinking to both D1 and Rap94, at different concentrations of VTF, is presented. The intensity was quantified and the average of three independent determinations is presented in Fig. 5D. As the amount of VTF is increased prior to irradiation, there is a 25% decrease in the ability of U9 to crosslink to Rap94. This result demonstrates that crosslinking of VTF and Rap94 to the termination motif is not mutually exclusive, but rather it supports a model in which both D1 and Rap94 interact with U9 in the ternary complex at the same time. The 25% reduction in crosslinking to Rap94 may be caused by the repositioning of the U9 motif on Rap94 after VTF binding reducing crosslinking efficiency.

Both VTF and Rap94 crosslink to an inactive mutant termination sequence

The sequence GUUUGUUUG is an altered termination motif that stimulates termination at less than 10% of the activity of the wild type sequence (data not shown). In order to determine whether the sequence alteration causes a decrease in the interaction of the mutant termination motif with either VTF or Rap94, ternary complexes were prepared on a mutant Ter29 kas template and crosslinking efficiency was evaluated as described above. In Fig. 5C the amount of transcript present in the wild type and mutant ternary complex demonstrates that the overall RNA level was comparable. In Figs. 5A,B, bottom panels, the crosslinking efficiency observed for both the D1 subunit and Rap94 is presented at varying amounts of added VTF. There is a clear reduction of crosslinking to both D1 and to Rap94 when the mutant RNA is compared to the wild type U9 sequence. This reduction is likely to be due both to the reduction in radioactive UMP in the U9

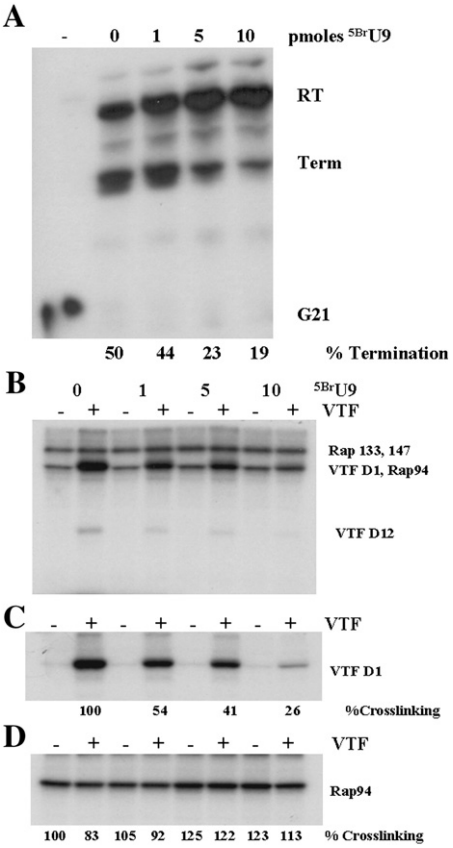

Fig. 6. A ^{5Br}U9 containing 22 base oligonucleotide inhibits both vaccinia virus early gene transcription termination and UV photo crosslinking to VTF. A. Addition of a ^{5Br}U9 oligonucleotide inhibits transcription termination in a standard bead-bound transcription termination assay. Lane 1, Step 1 G21 RNA. Step 2 transcription elongation of Step 1 RNA was done in the presence of four nucleoside triphosphates plus 2 pmol of VTF, in the absence or presence of various concentrations of ^{5Br}U9 oligonucleotide. RT, read through transcription to the end of the linear template. Term, the product of transcription termination in response to the UUUUUNU termination motif. The percentage of termination is presented at the bottom. The results represent the average of two independent determinations. B, C, D. UV photo crosslinking was carried out using Step 2 labeled ternary complexes incubated in the absence or presence of 2 pmol of VTF and various concentrations of ^{5Br}U9 oligonucleotide. B. Total UV photo crosslinking products were separated by denaturing gel electrophoresis and observed by autoradiography. C, D. UV photo crosslinked ternary complexes were denatured and either D1 or Rap94 proteins were collected by immunoprecipitation. The relative levels of radioactivity presented below each panel are the average of two independent determinations.

motif (6 versus 9) and the reduction of crosslinking efficiency when U is replaced by G. Importantly, when the level of crosslinking is considered as a percentage of maximal crosslinking for both proteins, there is no difference between the ternary complex containing the wild type U9 motif and the complex that harbors the termination motif mutant, Fig. 5D. The simplest interpretation is that the binding of VTF and Rap94 to the termination motif is not substantially altered by the introduction of the three G residues. Alternatively, any alteration in interaction with the mutant termination motif is comparable for both proteins.

^{5Br}U9 inhibition of VTF-dependent transcription termination correlates with inhibition of UV photo crosslinking to U9 in the isolated ternary complex

Prior studies showed that the addition of a 22 base oligonucleotide containing a ^{5Br}U9 sequence inhibited early gene transcription termination resulting in the synthesis of enhanced levels of read through transcript (Mohamed and Niles, 2003a,b). This contrasts sharply with studies that demonstrate that the addition of an oligonucleotide with a U9 motif stimulates premature early gene transcription termination. A competition test established that the addition of the ^{5Br}U9 oligonucleotide prevented U9 stimulation of premature transcription termination, verifying that the ^{5Br}U9 oligonucleotide interacts directly with U9 recognition factor. In an attempt to evaluate the functional significance of the observed interactions between both VTF and Rap94 with the U9 motif in the ternary complex, the effect of addition of a ^{5Br}U9 oligonucleotide on UV photo crosslinking was evaluated. The results in Fig. 6A demonstrate that addition of a 22 base ^{5Br}U9 oligonucleotide to an *in vitro* transcription reaction results in a reduction of transcription termination and the enhanced synthesis of the read through product. UV photo crosslinking was carried out with Step 2 radiolabeled ternary complexes in the presence or absence of added VTF and the absence or presence of increasing concentrations of the ^{5Br}U9 oligonucleotide. In Fig. 6B, it can be observed that the addition of the oligonucleotide has no affect on crosslinking to the largest RNA polymerase subunits. However, it appears that oligonucleotide addition reduces crosslinking both in the VTF D1/Rap94 region and the small VTF subunit D12 region of the gel. To distinguish between VTF D1 and Rap94, crosslinked samples were immunoprecipitated with polyclonal antibodies, Figs. 6C,D. It is apparent that the ^{5Br}U9 oligonucleotide dramatically reduces the ability of VTF D1 to crosslink to the U9 motif in the nascent RNA in the ternary complex. However, there is little indication of a reduction of crosslinking to Rap94. Rather, there appears to be an inverse relationship between crosslinking to D1 and to Rap94.

VTF interacts with the 5′ end region of the nascent RNA in a ternary complex

As an alternative approach, the ability of VTF to protect the nascent RNA in a ternary complex from RNAse A digestion was evaluated. Bead-bound ternary complexes were prepared at the end of Step 2 transcription on templates containing a wild type T9 sequence. The nascent RNA was either labeled in Step 1, which would limit the radioactivity to the sequence between U2 and U19, or labeled in Step 2, which would focus the label between U29 and U37 and between U60 and U76, Fig. 1B. The latter region resides within the RNA product channel in the RNA polymerase. Either RNA isolated from the ternary complex, or the intact ternary complex, was incubated in the presence or absence of VTF and further incubated with different levels of RNAse A. After digestion, RNA was isolated and separated by denaturing gel electrophoresis. Fig. 7B demonstrates that the free RNA is readily degraded at low RNAse A levels in the presence or absence of added VTF. Perhaps there is a low level of preferential protection in the presence of VTF. However, in the context of the isolated ternary complex, VTF affords substantial protection of the nascent RNA even at high RNAse A levels, Fig. 7A. Importantly, protection of the entire nascent RNA is observed, Fig. 7A, top panel. The VTF protection of the RNAse A cleavage of the 5′ region of the nascent RNA in isolated ternary complexes is apparent when quantified, in Fig. 7C.

This analysis was extended to an evaluation of VTF-dependent protection of ternary complexes radiolabeled in Step 2. Again, free Step 2 labeled RNA, isolated from bead-bound ternary complexes, is readily digested by RNAse A and VTF does not afford measurable protection to the transcript that is radiolabeled in the central and 3′ regions, Fig. 8B. When the labeled nascent RNA is present in an isolated ternary complex, however, VTF affords substantial protection at even the highest RNAse A level employed, Figs. 8A,C. Again, note that the full length transcript is protected. In addition, note that in the absence of VTF, RNAse cleavage of RNA in the ternary complex yields a nuclease insensitive fragment. This is the RNAse resistant portion in the 3′ region of the transcript that is protected by the RNA polymerase in the ternary complex.

In a final analysis, the ability of VTF to protect nascent RNA that contains either a wild type U9 termination motif or the mutant motif

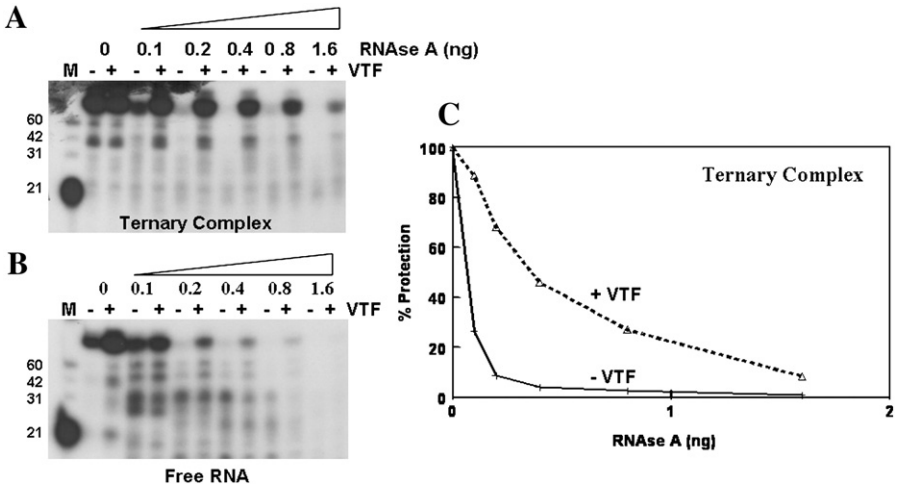

Fig. 7. VTF protects end labeled ternary complex RNA from RNAse A digestion. Step 2 ternary complexes were prepared containing transcripts radiolabeled in Step 1. Either RNA extracted from ternary complexes (B) or bead-bound ternary complexes (A) were incubated in the absence or presence of 3 pmol of VTF in the absence or presence of increasing amounts of RNAse A. After incubation at 37 °C, RNA was extracted, separated by gel electrophoresis and observed by autoradiography. Full length RNA was quantified in panel A by densitometry and the results were presented as % protection at different levels of RNAse A employed in the presence or absence of VTF (C). The results in C are the average of two independent determinations.

was evaluated. In this case a single concentration of RNAse A was employed that yielded an intermediate level of degradation of the transcript in the ternary complex. Isolated bead-bound ternary complexes prepared on wild type or mutant templates were incubated with different levels of VTF, digested with RNAse A and the isolated RNA products were evaluated by gel electrophoresis. The level of full length transcript was quantified and the results are presented in Figs. 9A,B,C. It is clear that for both templates, as the amount of VTF is elevated the level of protection of full length transcript is increased, Figs. 9A,B. However, the extent of protection for the nascent RNA that has the wild type U9 motif is not distinguishable from that with the mutant sequence supporting the prior observation that VTF interaction is not reduced by alteration of the termination motif. Included on the graph, Fig. 9C, is VTF-dependent transcription termination measured at different VTF concentrations. These results demonstrate that the level of VTF required for transcription termination is indistinguishable from that required to give protection from RNAse A.

Discussion

Termination of vaccinia virus early gene transcription is mediated by a novel pathway employing several viral proteins that respond to a unique sequence motif embedded in the nascent transcript. Termination utilizes both *trans*-acting factors and *cis*-acting components of the transcription ternary complex. The UUUUUNU termination motif lies in *cis* in the ternary complex, positioned outside of the RNA polymerase, available for interaction with one or more components of the termination pathway (Hagler and Shuman, 1992a). Rap94, the early gene specific RNA polymerase subunit is also located in the ternary complex, thus acting in *cis* in regards to transcription termination (Ahn et al., 1994; Deng and

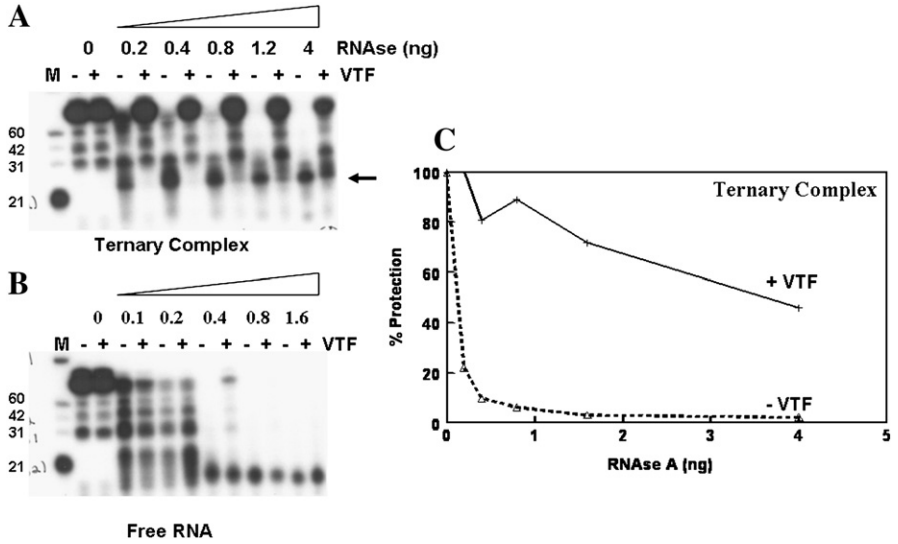

Fig. 8. VTF protects internally radiolabeled Step 2 RNA in an isolated ternary complex from RNAse A digestion. Preparation of ternary complexes, RNAse protection and analyses were performed as described in the legend to Fig. 7. A. RNA in isolated ternary complexes; B. RNA extracted from ternary complexes; and C. The quantification of the results in panel A is the average of two independent determinations. The arrow in A denotes the migration of the 3′ region of the nascent RNA protected by its location in the RNA product channel of the RNA polymerase. The fastest migrating component in A is a collection of short fragments produced by RNAse digestion.

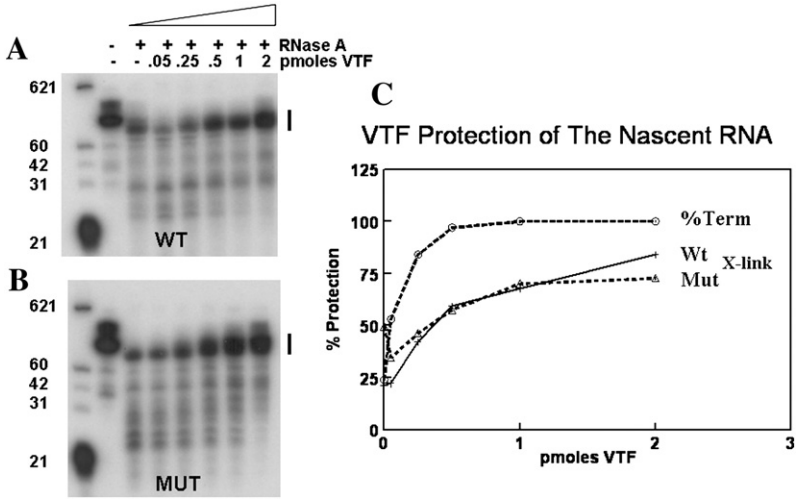

Fig. 9. VTF protection of nascent RNA in isolated ternary complexes is not affected by a mutation in the termination motif. Ternary complexes were prepared on bead-bound templates containing either a wild type (WT) U9 termination motif (A), or a mutant (Mut) termination motif (B). RNAse protection was tested in the absence or presence of increasing levels of RNAse A. RNA was extracted and separated by gel electrophoresis. The protected full length RNA, denoted by a bar, was observed by autoradiography and the amount was quantified by densitometry. The results are expressed in C as the percentage of full length RNA present after RNAse A digestion, at each concentration of VTF. Each point is the average of two independent determinations. In C, the percentage of transcription termination observed at each VTF concentration is also presented (o), (+) wild type termination motif, and (Δ) mutant termination motif.

Shuman, 1994). NPH I is present in the bead-bound isolated ternary complex and normally it is not necessary to be added to the reaction. However, in transcription competent extracts prepared from cells infected with a temperature sensitive mutation in gene D11L, transcription termination requires the addition of NPH I in *trans* (Christen et al., 1998). Thus, NPH I is part of the normal transcription elongation complex constructed on a bead-bound template. VTF, the vaccinia termination factor is easily separated from the ternary complex by salt wash of the bead-bound template. Both termination analysis and UV photo crosslinking demonstrate that a low level of VTF is retained in the washed ternary complex. Retention is somewhat variable and appears to be influenced by the template employed. Nonetheless, efficient termination requires VTF addition (Shuman et al., 1987). Prior studies from the Shuman laboratory showed that the ability to form a stable association of VTF and the ternary complex required an RNA chain length of greater than 31 nucleotides (Hagler and Shuman, 1992a; Hagler et al., 1994).

In principle, one or more of the ternary complex components might serve as the UUUUUNU recognition factor. The most likely candidates include the known termination factors, VTF, Rap94 and NPH I. However, this does not exclude either other subunits of the RNA polymerase, or a yet to be identified *trans*-acting termination factor. The use of the bead-bound Ter29 kas template provided a means to identify components of the ternary complex that interact with the UUUUUNU termination motif. UV photo crosslinking, in effect, traps proteins that lie close to the nascent RNA in the ternary complex. Digestion of the nascent RNA with ribonuclease T1, which cleaves after G, permits the retention of the entire U9 termination motif on the crosslinked protein. Crosslinks that occur outside of the radiolabeled region are not observed by this analysis. Importantly, label in the 3′ region of the Step 2 transcript crosslinks to the large RNA polymerase subunit, which serves as an effective and convenient internal control for these studies.

Initial experiments showed that the short, 21 base,G21 RNA transcript synthesized during Step 1 transcription crosslinked primarily to the largest RNA polymerase subunit. This demonstrates that the product RNA channel resides within the 146 kDa subunit in accord with the known structures of both bacterial and eukaryotic RNA polymerases (Gnatt et al., 2001) Addition of VTF revealed a low level of interaction between the D1 subunit and this short transcript. However, RNA of this length is not able to be capped *in vitro* indicating that a functional interaction between VTF and G21 RNA is not likely (Hagler and Shuman, 1992a).

Ternary complexes possessing a 77 base transcript labeled exclusively in Step 2 exhibit additional interactions. Since the transcript possesses radioactivity in the 3′ terminal region, crosslinking to the 146 kDa RNA polymerase subunit was anticipated. Additional interactions of the nascent transcript with the 133 kDa and 18 kDa RNA polymerase subunits were also observed. Importantly, crosslinking of U9 to Rap94, an RNA polymerase subunit known to be required both for early gene transcription initiation (Ahn et al., 1994; Deng and Shuman, 1994) and termination (Mohamed and Niles, 2001; Mohamed et al., 2002) was observed as well. Rap94 and the D1 subunit of VTF often co-migrate during SDS gel electrophoresis preventing prior identification of its interaction with the nascent transcript (Hagler et al., 1994). The functional significance of interactions of the 133 kDa and 18 kDa RNA polymerase subunits is not clear. These studies focused on U9 interactions with the known termination factors, VTF and Rap94, but we cannot exclude a role for either the 133 kDa or the 18 kDa RNA polymerase subunit in transcription elongation or termination.

The interaction of VTF with the nascent transcript in the isolated ternary complex is complicated by the fact that VTF also serves as the mRNA capping enzyme (Shuman et al., 1987). RNAse A digestion studies demonstrate weak protection of the 5′ region of an isolated transcript by VTF. However, in the context of the bead-bound ternary complex, VTF exhibits substantial protection of the exposed RNA. Importantly, VTF provides protection of the full length transcript demonstrating intimate contacts between VTF and the nascent RNA in the ternary complex not observed with the free RNA. In a ternary complex containing an internally radiolabeled transcript, VTF also exhibits extensive protection of the entire RNA polymerase-bound transcript. These results support a model in which the nascent transcript resides sandwiched between VTF and the RNA polymerase in an isolated ternary complex. This is consistent with the requirement for an RNA of 51 nucleotides in length to permit formation of a stable complex between VTF and the RNA polymerase in the ternary complex (Hagler et al., 1994). The site of UV photo crosslinking of VTF to RNA in the ternary complex maps to the N-terminal domain of the large D1 subunit of VTF. An RNA binding site was identified previously in the same domain (Myette and Niles, 1996b). Interestingly, a single base mutation in the termination motif, known to reduce termination efficiency by 90% does not exhibit a corresponding reduction in crosslinking. This indicates that the loss of termination activity in this

mutation is not due simply to reduced binding between VTF and the termination motif. Rather, it seems likely that the mutation prevents the action resulting from the VTF/UUUUUNU interaction.

Of the four RNA polymerase subunits that crosslink to internally radiolabeled RNA, Rap94 is the most interesting. Two lines of evidence demonstrate that Rap94 interacts with U9 rather than the 3′ labeled region of C77 RNA, U60 to U76. First, Rap94 is not labeled in ternary complexes containing radiolabeled G21 RNA, which contains the same number and distribution of U residues as found in the 3′ region of Step 2 RNA. Second, the labeling efficiency of Rap94 is reduced when ternary complexes contain C77 RNA harboring a mutant termination motif. Rap94 is known to be an essential early gene transcription termination factor (Mohamed and Niles, 2001; Mohamed et al., 2002). In addition, the N-terminal end of Rap94 binds to the C-terminal end of NPH I, which tethers NPH I to the RNA polymerase in the ternary complex (Mohamed and Niles, 2000). This interaction also provides a likely reason for the restriction of U5NU motif dependent transcription termination to early genes since the RNA polymerase that transcribes intermediate and late genes lacks the Rap94 subunit. The interaction between Rap94 and the U5NU termination motif is not prevented by a mutation in the termination sequence indicating that reduced binding to Rap94 does not explain the reduced termination activity exhibited by this mutation. Furthermore, crosslinking of U5NU to Rap94 is not dependent upon the presence of VTF since maximal crosslinking is observed in salt washed ternary complexes from which VTF has been removed. However, addition of VTF causes a dose dependent reduction of crosslinking to Rap94 but the decrease is limited to about 25%, at saturating VTF. This indicates that both VTF and Rap94 can interact with U9 at the same time, which supports the sandwich model described above.

Addition of a ^{5Br}U9 22 base oligonucleotide inhibits both transcription termination and UV photo crosslinking. The loss of termination activity correlates well with a decrease in crosslinking to both the large and small subunit of VTF. These data do not permit us to determine whether the oligonucleotide interacts directly with VTF bound in the ternary complex or with VTF that has dissociated from the ternary complex and is prevented from reassociating with the U9 motif. In spite of this ambiguity, the observed correlation provides strong support for a direct functional interaction between both subunits of VTF and the termination motif in the isolated ternary complex. Interestingly, crosslinking to Rap94 is not reduced by ^{5Br}U9 addition. Rather, there is a modest increase in crosslinking due to the increase in VTF-free transcript in the ternary complex. The molecular details of the interactions among the U5NU termination motif and both VTF and Rap94 are likely to provide the insight essential for defining these initial steps in transcription termination.

Our current understanding of early gene transcription termination is presented in cartoon form in Fig. 10. A termination competent ternary complex contains the RNA polymerase bound to a bubble in the template that has the 3′ end of the nascent transcript annealed to the template strand. The RNA chain extends through the product channel in the 146 kDa subunit for about 18 bases (Hagler and Shuman, 1992b). Upon exit from the channel, the transcript can interact with additional RNA polymerase subunits, including Rap94. When VTF is present, VTF interacts extensively with the transcript, including both the 5′ end region and the central sequence containing the U5NU termination motif. In our studies, there is no guarantee that a single VTF molecule is responsible for this entire interaction. However, it is clear that the full protection requires that the transcript reside in the ternary complex since it is not observed when free RNA is tested. The most parsimonious model posits that the transcript lies sandwiched between VTF and the RNA polymerase. This is supported by an apparent simultaneous interaction between UUUUUNU, VTF and the RNA polymerase subunits, notably Rap94. NPH I is a single stranded DNA-dependent ATPase that provides the energy required for termination (Deng and Shuman, 1998; Christen et al., 1998). Since

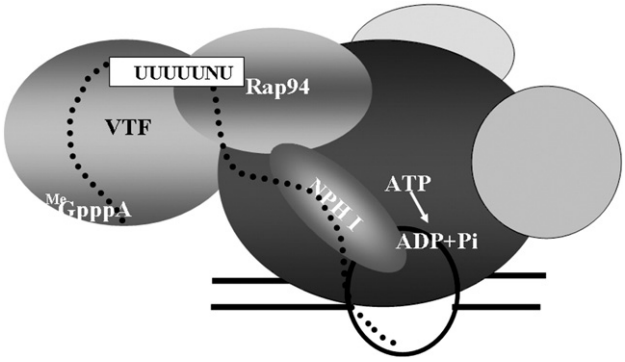

Fig. 10. A model of a transcription ternary complex prior to termination and transcript release. The nascent RNA is represented by a dotted line. The relevant subunits are labeled: VTF, vaccinia termination factor; Rap94, the early gene transcription specific RNA polymerase subunit; and NPH I, nucleoside triphosphate phosphohydrolase I, which catalyzes the hydrolysis of ATP and provides the energy required for transcript release. The other objects represent other subunits of the viral RNA polymerase.

ssDNA is required to reveal a normally cryptic active site, the model shows NPH I binding to the non-template strand in the bubble. Furthermore, the C-terminal end of NPH I must interact with the N-terminal domain of Rap94 for termination to occur (Mohamed and Niles, 2000, 2001, Piacente et al., 2003). This overall arrangement of factors in the termination competent ternary complex argues that the interaction of VTF with the U5NU termination motif and Rap94 initiates the transmission of the termination command through Rap94 to NPH I, which now is stimulated to hydrolyze ATP and provide the impetus to terminate transcription and release the nascent transcript.

Materials and methods

Transcription assays

A plasmid containing the chimeric gene Ter29 (Deng et al., 1996) was generously provided by Dr. Stewart Shuman of Memorial Sloan Kettering Cancer Center. Ter29 was constructed to serve as a tool to evaluate vaccinia virus early gene transcription termination and transcript release, *in vitro*, Fig. 1A. The GC rich sequence at the beginning of the A-less cassette was changed into a Kas I recognition site. The altered gene is referred to as Ter 29 kas. PCR was employed to amplify the Ter29 segment from the plasmid. One primer contained biotin linked to the 5′ end, while the other primer was phosphorylated by bacteriophage T4 polynucleotide kinase with γ^{32}P ATP of known specific activity. The biotinylated linear double stranded DNA segment was bound to strepavidin beads and employed in transcription assays. The linear DNA fragment contains a strong early promoter followed by a 20 base pair G-less cassette, a 57 base pair A-less cassette and a 112 base pair segment of random sequence. Transcription takes place in a series of steps. In Step 1, transcription was conducted in the absence of GTP and the presence of 3′ *O* methyl GTP through a 20 base pair G-less cassette, yielding a 21 base transcript, G21 RNA. Bead-bound ternary complexes were collected using a magnet, washed and resuspended in transcription assay salts. Step 2 transcription was conducted in two ways. First, transcription termination was evaluated by elongation of the G21 RNA in the presence of 1 mM of all four nucleoside triphosphates plus the addition of the vaccinia termination factor, VTF. Failure to terminate yields a 189 base runoff transcript. VTF-dependent termination produces a shortened transcript of about 75 bases in length, Fig. 1A. Second, for both UV photo crosslinking experiments and for ribonuclease A protection studies Step 2 transcription was conducted in the absence of ATP and VTF. This permitted elongation of G21 RNA through the adjacent A-less cassette up to the first AT base pair at position 78. This yields a stable ternary complex that contains a 77 base RNA, C77. Importantly, the U9

termination motif resides at a position in the transcript located outside of the RNA polymerase and available for interacting with U9 recognition proteins (Hagler and Shuman, 1992b). Step 1 transcription was conducted at 30 °C for 10 min in 20 mM Tris HCl, pH 8.0, 6 mM $MgCl_2$, 100 fmol bead-bound Ter29 template 1 mM CTP, 1 mM ATP, 0.5 μCi α^{32}P UTP, and 0.25 mM 3′ *O* methyl GTP. Beads were collected, washed with 20 μl 0.25 M potassium acetate and three times in 20 μl of transcription salts and resuspended in transcription salts. For transcription termination assays, Step 2 transcription was carried out in transcription assay salts, 1 mM NTPs plus or minus 2 pmol of VTF. RNA samples were separated on 11% polyacrylamide gels containing 8 M urea.

Enzyme preparation

Vaccinia virus was grown (Condit and Motyczka, 1981) and purified (Baroudy and Moss, 1980), as described. Early gene transcription competent RNA polymerase was prepared from sucrose gradient purified virus through two DEAE cellulose column chromatography steps as described (Baroudy and Moss, 1980; Shuman et al., 1987). For some experiments, the RNA polymerase was size-fractionated on a glycerol gradient (Shuman et al., 1987). RNA polymerase was aliquoted and stored at −80 °C without loss of activity. VTF, D^{1-545}, $D1^{498-844}$/D12 and NPH I were prepared from *E. coli* engineered to over express each enzyme, as described (Higman et al., 1992, 1994; Myette and Niles, 1996a).

UV photo crosslinking

The sequence of the Ter29 template was modified to remove a group of Ts from the non-template strand between positions 41 and 57 and replaced with C or G, Fig. 1B. The plasmid was constructed by ligating two synthetic double stranded DNA fragments to pGem3Zf+ cleaved with Sal I and Xba I. The left fragment contained the strong early promoter region and the G-less cassette flanked on the left end with a Sal I overhang and on the right end with a Kas I overhang. The right fragment contained the remainder of the Ter29 sequence through the A4 region in nucleotides 78 to 81. This fragment was flanked by a Kas I overhang on the left end and an Xba I overhand on the right end. The two fragments were joined and inserted into the plasmid in a three way ligation. The DNA sequence of each insert was determined. Bead-bound ternary complexes were prepared at the end of Step 1 or Step 2 transcription. For UV photo crosslinking experiments glycerol gradient purified RNA polymerase was employed. Due to the small volume of each fraction the optical density of the RNA polymerase preparation was not determined. The enzyme level used in each set of experiments was determined by a preliminary titration of each enzyme preparation and assaying the extent of crosslinking. Up to 3 μl of gradient purified RNA polymerase was employed in each 20 μl reaction. The Step 1 ternary complexes were constructed by transcription in the presence of 1 mM CTP, 1 mM ATP, 0.375 mM 3′ *O* methyl GTP and 10 μCi α^{32}P UTP for 10 min at 30 °C. Beads were collected, washed and resuspended in transcription salts. Step 2 ternary complex located at the end of the A-less cassette were radiolabeled during Step 2 transcription. G21 RNA containing Step 1 ternary complexes were prepared in the absence of radiolabeled UTP and the presence of 0.1 mM UTP. Step 2 transcription was conducted in the presence of 2 μM UTP, 1 mM CTP, 1 mM GTP and 10 μCi α^{32}P UTP for 20 min at 30 °C. Bead-bound ternary complexes were aliquoted into 96 well dishes and incubated in the presence or absence of VTF, or VTF subdomains, VTF $D1^{1-545}$ and VTF $D1^{498-844}$/D12, on an ice water bath. A germicidal lamp was placed 10 cm above the dish and the samples were irradiated for 20 min. After irradiation, samples were treated with 1000 U of ribonuclease T1, and 1 U of deoxyribonuclease I, denatured by the addition of a 1/3 volume of 4× protein sample buffer, boiled and analyzed by SDS gel electrophoresis on 10% polyacrylamide gels. For immunoprecipitation analysis, irradiated samples were adjusted to 0.5% SDS and 10 mM β-mercaptoethanol and boiled to denature the complexes. Immunoprecipitations were carried out as previously described (Niles and Seto, 1988; Christen et al., 1992). Polyclonal antibodies directed against the RNA polymerase subunits were generously provided by Dr. Stewart Shuman. Antibodies directed against VTF, Rap94, and NPH I were described (Higman et al., 1992; Mohamed et al., 2002; Christen et al., 1992, 1998).

^{5Br}U9 competition studies

Step 2 radiolabeled ternary complexes were prepared as described above. Complexes were incubated in the absence or presence of 2 pmol of VTF and the absence or presence of a 22 base ribo oligonucleotide containing a central ^{5Br}U9 motif. UV photo crosslinking was done as written above in the presence of varying amounts of oligonucleotide. Transcription assays were conducted in the presence of 2 pmol of VTF and the presence of varying levels of ^{5Br}U9 oligonucleotide (Mohamed and Niles, 2003a,b). Transcription termination was evaluated after separation of the transcripts by gel electrophoresis, autoradiography and densitometric analysis of the exposed film.

Ribonuclease A protection studies

Step 2 ternary complexes located at the end of the A-less cassette, radiolabeled during either Step 1 or Step 2 transcription, were prepared as described above. Complexes were washed, resuspended and aliquoted into eppendorf tubes for ribonuclease A treatment. As a control, RNA was isolated from the bead-bound ternary complexes and incubated directly. Both bead-bound ternary complexes and free RNA were incubated with VTF for 20 min, at 30 °C. Varying amounts of ribonuclease A were added and samples were incubated for an additional 15 min. Ribonuclease A resistant RNA was extracted, resuspended in formamide, heated and analyzed by denaturing urea gel electrophoresis on 11% polyacrylamide gels. RNA fragments were observed by autoradiography and RNA levels were quantified by densitometry.

Acknowledgment

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References

Ahn, B.Y., Moss, B., 1992. RNA polymerase-associated transcription specificity factor encoded by vaccinia virus. Proc. Natl. Acad. Sci. U. S. A. 89, 3536–3540.
Ahn, B.Y., Gershon, P.D., Moss, B., 1994. RNA polymerase-associated protein Rap94 confers promoter specificity for initiating transcription of vaccinia virus early stage genes. J. Biol. Chem. 269, 7552–7557.
Baroudy, B.M., Moss, B., 1980. Purification and characterization of a DNA-dependent RNA polymerase from vaccinia virions. J. Biol. Chem. 255, 4372–4380.
Broyles, S.S., 2003. Vaccinia virus transcription. J. Gen. Virol. 84, 2293–2303.
Christen, L.A., Higman, M.A., Niles, E.G., 1992. Phenotypic characterization of three temperature sensitive mutations in the vaccinia virus early gene transcription initiation factor. J. Gen. Virol. 73, 3155–3167.
Christen, L.A., Sanders, M., Wiler, C., Niles, E.G., 1998. Vaccinia virus nucleoside triphosphate phosphohydrolase I is an essential viral early gene transcription termination factor. Virology 245, 360–371.
Christen, L., Sanders, A., Niles, E.G., 1999. Interaction of the vaccinia virus nucleoside triphosphate phosphohydrolase I with linear oligonucleotides. Biochemistry 36, 8072–8089.
Condit, R.C., Motyczka, A., 1981. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. Virology 113, 224–241.
Condit, R.C., Lewis, J.I., Quinn, M., Christen, L.M., Niles, E.G., 1996. Use of lysolecithin-permeabilized infected-cell extracts to investigate the in vitro biochemical phenotypes of poxvirus ts mutations altered in viral transcription activity. Virology 218, 169–180.
Deng, L., Shuman, S., 1994. A role for the H4 subunit of vaccinia RNA polymerase in transcription initiation at a viral early promoter. J. Biol. Chem. 269, 14323–14328.

Deng, L., Shuman, S., 1996. An ATPase component of the transcription elongation complex is required for factor-dependent transcription termination by vaccinia RNA polymerase. J. Biol. Chem. 271, 29386–29392.
Deng, L., Shuman, S., 1998. Vaccinia NPH-I, a DExH-box ATPase, is the energy coupling factor for mRNA transcription termination. Genes Dev. 12, 538–546.
Deng, L., Hagler, J., Shuman, S., 1996. Factor-dependent release of nascent RNA by ternary complexes of vaccinia RNA polymerase. J. Biol. Chem. 271, 19556–19562.
Ensinger, M.J., Martin, S.A., Paoletti, E., Moss, B., 1975. Modification of the 5′-terminus of mRNA by soluble guanylyl and methyl transferases from vaccinia virus. Proc. Natl. Acad. Sci. U. S. A. 72, 2525–2529.
Gnatt, A.L., Cramer, P., Fu, J., Bushnell, D.A., Kornberg, R., 2001. Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 A resolution. Science 292, 1876–1882.
Hagler, J., Shuman, S., 1992a. A freeze-frame view of eukaryotic transcription during elongation and capping of nascent mRNA. Science 255, 983–986.
Hagler, J., Shuman, S., 1992b. Structural analysis of ternary complexes of vaccinia RNA polymerase. Proc. Natl. Acad. Sci. U. S. A. 89, 10099–10103.
Hagler, J., Luo, Y., Shuman, S., 1994. Factor-dependent transcription termination by vaccinia RNA polymerase. Kinetic coupling and requirement for ATP hydrolysis. J. Biol. Chem. 269, 10050–10060.
Higman, M.A., Bourgeois, N., Niles, E.G., 1992. The vaccinia virus mRNA (guanine-N7-)-methyltransferase requires both subunits of the mRNA capping enzyme for activity. J. Biol. Chem. 267, 16430–16437.
Higman, M.A., Christen, L.A., Niles, E.G., 1994. The mRNA (guanine-7-) methyltransferase domain of the vaccinia virus mRNA capping enzyme. Expression in *Escherichia coli* and structural and kinetic comparison to the intact capping enzyme. J. Biol. Chem. 269, 14974–14981.
Hooda-Dhingra, U., Thompson, C.L., Condit, R.C., 1989. Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. J. Virol. 63 (2), 714–729.
Kane, E., Shuman, S., 1992. Temperature-sensitive mutations in the vaccinia virus H4 gene encoding a component of the virion RNA polymerase. J. Virol. 66, 5752–5762.
Luo, Y., Shuman, S., 1993. RNA binding properties of vaccinia virus capping enzyme. J. Biol. Chem. 268, 21253–21262.
Mohamed, M.R., Niles, E.G., 2000. Interaction between nucleoside triphosphate phosphohydrolase I and the H4L subunit of the viral RNA polymerase is required for vaccinia virus early gene transcript release. J. Biol. Chem. 275, 25798–25804.
Mohamed, M.R., Niles, E.G., 2001. The viral RNA polymerase H4L subunit is required for vaccinia virus early gene transcription termination. J. Biol. Chem. 276, 20758–20765.
Mohamed, M.R., Niles, E.G., 2003a. UUUUUNU oligonucleotide stimulation of vaccinia virus early gene transcription termination, *in trans*. J. Biol. Chem. 278, 11794–11801.
Mohamed, M.R., Niles, E.G., 2003b. UUUUUNU stimulation of vaccinia virus early gene transcription termination: oligonucleotide sequence and structural requirements for stimulation of premature termination, *in vitro*. J. Biol. Chem. 278, 39534–39541.
Mohamed, M.R., Christen, L., Niles, E.G., 2002. Antibodies directed to an epitope in the H4L subunit of the vaccinia virus RNA polymerase inhibit both active preinitiation complex formation and transcription termination activity. Virology 299, 142–153.
Moss, B. Ed. 2007. Poxviridae: the viruses and their replication, vol. 2. Edited by D.Ml. Knipe, P.M. Howley, D.E. Griffin, R.A. Lamb, M. A. Martin, B. Roizman, S.E. Strauss, 2 vols. Philadelphia: Lippincott-Raven.
Myette, J.R., Niles, E.G., 1996a. Characterization of the vaccinia virus RNA 5′-triphosphatase and nucleotide triphosphate phosphohydrolase activities. Demonstrate that both activities are carried out at the same active site. J. Biol. Chem. 271, 11945–11952.
Myette, J.R., Niles, E.G., 1996b. Domain structure of the vaccinia virus mRNA capping enzyme. Expression in *Escherichia coli* of a subdomain possessing the RNA 5′-triphosphatase and guanylyltransferase activities and a kinetic comparison to the full-size enzyme. J. Biol. Chem. 271, 11936–11944.
Niles, E.G., Seto, J., 1988. Vaccinia virus gene D8 encodes a virion trans-membrane protein. J. Virol. 62, 3772–3778.
Paoletti, E., Moss, B., 1974. Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus. Nucleotide substrate and polynucleotide cofactor specificities. J. Biol. Chem. 249, 3281–3286.
Piacente, S.C., Christen, L.M., Mohamed, M.R., Niles, E.G., 2003. Effect of selected mutations in the C-terminal region of the vaccinia virus nucleoside triphosphate phosphohydrolase I (NPH I) on binding to the H4L subunit of the viral RNA polymerase and early gene transcription termination. Virology 310, 109–117.
Rohrmann, G., Yuen, L., Moss, B., 1986. Transcription of vaccinia virus early genes by enzymes isolated from vaccinia virions terminates downstream of a regulatory signal. Cell 46, 1029–1035.
Shuman, S., Morham, S.G., 1990. Domain structure of vaccinia virus mRNA capping enzyme. Activity of the Mr 95,000 subunit expressed in *Escherichia coli*. J. Biol. Chem. 265 (20), 11967–11972.
Shuman, S., Moss, B., 1988. Factor-dependent transcription termination by vaccinia RNA polymerase: Evidence for a *cis*-acting termination signal in nascent RNA. J. Biol. Chem. 263, 6220–6225.
Shuman, S., Broyles, S.S., Moss, B., 1987. Purification and characterization of a transcription termination factor from vaccinia virions. J. Biol. Chem. 262, 12372–12380.
Venkatesan, S., Gershowitz, A., Moss, B., 1980. Modification of the 5′ end of mRNA. Association of RNA triphosphatase with the RNA guanylyltransferase-RNA (guanine-7-) methyltransferase complex from vaccinia virus. J. Biol. Chem. 255, 903–908.
Vos, J.C., Sasker, M., Stunnenberg, H.G., 1991. Vaccinia virus capping enzyme is a transcription initiation factor. EMBO J. 10, 2553–2558.
Wright, C.F., Coroneos, A.M., 1995. The H4 subunit of vaccinia virus RNA polymerase is not required for transcription initiation at a viral late promoter. J. Virol. 69, 2602–2604.
Yuen, L., Moss, B., 1986. Multiple 3′ ends of mRNA encoding vaccinia virus growth factor occur within a series of repeated sequences downstream of T clusters. J. Virol. 60, 320–323.
Yuen, L., Moss, B., 1987. Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. Proc. Natl. Acad. Sci. U. S. A. 84, 6417–6421.