UUUUUNU Stimulation of Vaccinia Virus Early Gene Transcription Termination

OLIGONUCLEOTIDE SEQUENCE AND STRUCTURAL REQUIREMENTS FOR STIMULATION OF PREMATURE TERMINATION IN VITRO

Received for publication, June 9, 2003, and in revised form, July 29, 2003
Published, JBC Papers in Press, July 30, 2003, DOI 10.1074/jbc.M306048200

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Vaccinia virus early genes are unique in that transcription terminates in a signal- and factor-dependent manner. Recent results from this laboratory demonstrated that a 22-mer RNA oligonucleotide containing a central U9 sequence exhibited sequence- and concentration-dependent stimulation of premature transcription termination and transcript release in trans. In an effort to better understand the different aspects of the USNU stimulation of premature termination, we evaluated the activity of various oligonucleotides in vitro. Neither RNA containing a mutant USNU signal nor single-stranded DNA containing T5NT was able to stimulate premature termination, demonstrating both sequence specificity and a requirement for ribose. Furthermore, neither oligonucleotide was able to compete with USNU, demonstrating that each failed to bind to the USNU recognition factor. Substitution of the U9 signal with either BrU9 or BrdU9 inhibited normal termination but did not stimulate premature termination. The addition of BrdU5NdU inhibited USNU stimulation of premature termination, demonstrating that both oligonucleotides bind to the same site on the USNU recognition factor. Finally, USNU containing RNA as short as nine bases served as an effective stimulator of premature termination. These observations impact directly on the development of oligonucleotide-based anti-poxvirus therapeutic agents.

Vaccinia virus, the prototypic poxvirus, possesses a double-stranded DNA genome of 191,686 base pairs (1) capable of encoding ~200 proteins. Poxviruses replicate within the cytoplasm of the infected cell. Their partial independence from host cell nuclear functions is aided by a distinctive replication and transcription apparatus encoded by viral genes (for review, see Ref. 2).

Vaccinia virus genes are expressed in a cascade that is divided into three temporal classes: early, intermediate, and late. The multisubunit viral RNA polymerase requires separate and non-overlapping sets of auxiliary proteins to initiate transcription of each gene class (3). Early genes are transcribed in the virus core immediately upon infection. Virions possess two forms of RNA polymerase that differ in subunit composition (4, 5). The one that is active in early gene transcription has one extra subunit, Rap94, the product of gene H4L (6). Shut off of early gene transcription accompanies the onset of DNA replication. Intermediate genes are transcribed in the cytoplasm subsequent to DNA replication and require prior early gene expression (7, 8). Late gene transcription follows the intermediate class and requires the synthesis of three intermediate proteins (9). Host factors are also employed in both intermediate (10) and late (11, 12) mRNA synthesis.

Early viral genes are unique in that transcription terminates in a signal- and factor-dependent manner (13–15). During infection, signal-dependent transcription termination is restricted to early genes. In vitro, only RNA polymerase capable of recognizing early promoters is subject to signal-dependent termination, indicating that this form of RNA polymerase is uniquely termination-competent (16). Termination requires the virion form of RNA polymerase, containing the Rap 94 subunit (17–19), and the vaccinia termination factor, VTF, which also serves as viral mRNA capping enzyme (14). In addition, nucleoside triphosphate phosphohydrolase I (NPH I), the product of D11L gene, serves as the ATPase employed in transcription termination (20, 21). An interaction between the C-terminal end of NPH I and the N-terminal end of Rap 94 is required for termination (17–19, 22). Elongation proceeds through the sequence TTGTNT in the nontemplate strand, yielding UUUUNU in the nascent mRNA, which serves as a signal required for the termination event (23, 24). The sequence UUUUNU must be at least 30 nucleotides from the 3′ end, indicating that it is recognized “outside” of the RNA polymerase (25). This suggests that the USNU signal interacts with an undefined essential termination factor, and upon binding, this factor initiates the termination/release sequence of events.

Recently, experiments were conducted in this laboratory in an attempt to identify the USNU binding factor and to ascertain its role in termination. Surprisingly, the addition of a USNU-containing oligonucleotide to a transcription reaction in vitro stimulated premature termination and transcript release rather than simply inhibiting termination. Stimulation was shown to be dependent on the presence of VTF, NPH I, and ATP or dATP and inhibited by antibodies directed against Rap 94, demonstrating the involvement of the normal transcription termination machinery (26). In this report we present the results of these studies.
Oligonucleotide Stimulation of Premature Termination

Several further studies on the oligonucleotide sequence and structural features required to stimulate premature transcription termination. We show that neither RNA containing a mutant U5NU signal nor ssDNA containing T5NT is able to stimulate premature termination, demonstrating both sequence specificity and a requirement for ribose. In addition, a dU9-containing RNA/DNA oligonucleotide chimera fails to stimulate premature termination, confirming the requirement for ribose in the signal sequence. Moreover, substitution of the U9 signal with either BrU9 or BrdU9 inhibits normal termination but does not stimulate premature termination. Furthermore, BrdU5NdU serves as competitive inhibitor of U5NU-dependent stimulation of premature termination and transcript release, demonstrating that both oligonucleotides bind to the same site on the U5NU recognition factor. Finally, U5NU RNA as short as 9 bases serves as an effective stimulator of premature termination. These observations impact directly on the design of oligonucleotide based anti-virus therapeutic agents.

EXPERIMENTAL PROCEDURES

Cells and Viruses—Propagation of the wild type (WT) vaccinia virus strain WR and the temperature-sensitive mutant virus, tsC50, was carried out in BSC40 African green monkey cells at 37 °C or the permissive temperature for ts mutants, 31 °C, respectively, as described (28, 29). Virus titers were determined by plaque formation on BSC40 cells at the permissive temperature, 31 °C, and the nonpermissive temperature, 40 °C, for tsC50 virus and at 37 °C for the WT virus.

Transcription Extracts—Preparation of transcription extracts from virus-infected cells by lysolceithin treatment was described previously (18, 21). Briefly, A549 cells were infected with either WT virus or tsC50 mutant virus at a multiplicity of infection of 15, at 31 °C. After 24 h, the medium was replaced with 40 °C medium containing 100 μg/ml cycloheximide and further incubated for another 24 h at 40 °C. The cells were washed and treated with 250 μg/ml lysolceithin, and extracts were prepared. In the case of the tsC50 virus-infected cells, this procedure permits the initial synthesis of active NPH I, which is required for intermediate and late gene expression. After switching to 40 °C, the endogenous NPH I is inactivated, and cycloheximide prevents the synthesis of new protein.

Oligonucleotides—Oligonucleotides used in this study were either chemically synthesized by Integrated DNA Technology Inc. (IDT) or synthesized in vitro. The large scale in vitro synthesis of the two 36-mer oligonucleotides, pGEM-U5NU and pGEM-BrU5NU, was carried out using a procedure that was previously described by Higman et al. (29) and Myette and Niles (30). Briefly, RNA was synthesized by the transcription of linearized plasmid DNA template by T7 RNA polymerase. To produce the 36-nucleotide-long transcripts, the following plasmid was constructed. An oligonucleotide with the sequence AATTGGGCCG-GCTTTTTTTTTTGCGTTG was synthesized and annealed to an oligonucleotide containing AATT-5’ overhangs.

5’-AATGGGCGGCTTTTTTTTGCGTTG-3’

3’-CCGGGGCGAAAAAAACGCAACTTA-5’

SEQUENCE 1

This fragment was ligated into the EcoRI site of pGEM3Zf (-), and the orientation was determined by sequencing the DNA. The downstream end retains the EcoRI recognition site so that cleavage with EcoRI yields a linear template used in run-off transcription. Transcription of the plasmid linearized at the EcoRI site yielded a 32-mer oligonucleotide with the sequence pggGGCGGGAUUUGGGCCGGUUG-UUUUUGCGGUGAU-AU. Transcription was carried out in the presence of either UTP or bromo-UTP to synthesize either pGEM-U5NU or pGEM-BrU5NU oligonucleotides, respectively. One-ml transcription reactions contained 200 μg of linearized plasmid DNA, 10 mM dithiothreitol, 1 mM ATP, CTP, GTP, and UTP or bromo-UTP and 500 units of T7 RNA polymerase. To facilitate the detection of RNA during purification, 2.5 μCi of [32P]UTP was included in each reaction. Transcription reactions were carried out for 2 h at 37 °C. The reactions were quenched, and protein was extracted by adding an equal volume of 24:24:1 phenol-stE (150 mM sodium chloride, 10 mM Tris HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid)-chloroform-isooctyl alcohol. The RNA was precipitated with 2.5 x ammonium acetate and 3 volumes of 95% ethanol. Transcription products were separated by electrophoresis on 10% acrylamide, 8 M urea gels and excised from the wet gels using an autoradiograph as a guide. RNA was eluted from the gel, precipitated with 0.3 M sodium acetate and three volumes of ethanol, dried, and resuspended in 250 μl of H2O.

Transcription Using a Bead-bound Template—Construction of the G21(TER29)A78 template was described previously (25), and the plasmid containing this sequence was generously provided by Dr. Stewart Shuman of the Memorial Sloan Kettering Cancer Center. The G21(TER29)A78 transcription unit contains a synthetic early promoter fused to a 20-nucleotide G-less cassette, which is followed by 3 G residues at positions +21 to +23. RNA synthesis in the absence of GTP and the presence of 3’ O methyl GTP yields a 21-nucleotide product that can be identified by gel electrophoresis. Downstream of the G-less cassette lies a 57-nucleotide A-less cassette that is flanked at its 3’ end by 4 A residues at positions +78 to +81. A termination signal, TTTTTTTTTT, lies within the A-less cassette, spanning position +29 to +37. The biotinylated 324-nt DNA template was amplified by PCR employing a 5’-biotinylated downstream primer and isolated by preparative agarose gel electrophoresis. The purified DNA fragment was then immobilized to streptavidin-coated magnetic beads (Dynabeads M280; Dynal) as described (31).

The bead-bound (B) template (typically 100 fmol) was first incubated with 10 μCi of [32P]CTP (800 Ci/mmol), 0.5 μM CTP, 0.1 mM UTP, and 0.5 mM 3’-Ome-GTP to synthesize the G21 transcript. The radio-labeled ternary complex was isolated, washed twice with 0.5 ml of transcription salts, resuspended, and incubated in the presence or absence of VTF, which was preincubated in the presence or absence of the oligonucleotide for 10 min on ice. Termination was then assessed after elongation of the ternary complex in the presence of 1 mM ATP, 1 mM GTP, 1 mM CTP, and 1 mM ATP. The 3’-Ome-GMP nucleotide was removed from the 3’ end of the G21 RNA by the intrinsic transcript cleavage activity of the vaccinia RNA polymerase before elongation (31).

Elongation of the G21 RNA to the end of the template would yield a transcript through transcription (RT) of about 177 nucleotides. Normal U5NU-dependent termination would yield a termination product of about 75 nucleotides in length. Greater than 90% of the isolated ternary complexes were routinely elongated in the second RNA synthesis reaction. RNA products were separated by gel electrophoresis, observed by autoradiography, and quantified by densitometry of the exposed film. Termination efficiency was calculated as the molar ratio of terminated RNA to the sum of read-through and terminated RNA. Premature termination efficiency was calculated as the molar ratio of premature terminated RNA to the sum of read-through, terminated, and premature terminated RNA.

Transcript Release from the G21 Ternary Complex—Bead-bound ternary complexes containing radio-labeled G21 RNA were constructed as described above using a tsC50 virus-infected cell extract. The ternary complex was isolated, washed, and resuspended. Transcript release from the paused ternary complex was assessed in the presence of VTF, NPH I, and dATP or the presence and absence of both the U5NU oligonucleotide and increasing concentrations of the BrdU5NU oligonucleotide. After incubation for 10 min at 30 °C, the bound transcript was separated from the free RNA using a magnet and quantified after gel electrophoresis as described above.

RESULTS

Prior studies conducted in this laboratory demonstrated that UUUUUNU-containing oligonucleotides stimulate premature termination and transcript release (26). The observed stimulation required viral proteins Rap94, VTF, NPH I, and dATP, demonstrating the involvement of the normal transcription termination machinery (26). To further investigate this phenomenon we evaluated the ability of different oligonucleotides to stimulate premature termination in vitro (Fig. 1). Both U5NU (22-mer) and mutant U5NU were described previously (26). pGEM-U5NU and pGEM-BrU5NU are 36-mer RNA oligonucleotides that were synthesized in vitro with UTP or bromo-UTP by T7 RNA polymerase and contain a U9 or a BrU9 signal, respectively. Both dU5NU and BrdU5NdU are 22-mer RNA/DNA chimeras that are identical to U5NU (22-mer) except for the substitution of the U9 signal sequence with either...
Oligonucleotide Stimulation of Premature Termination

Both USNU (22-mer) and mutant-USNU were previously described (26). The pGEM-U5NU and the pGEM-BrU5NU are 36-mer RNA oligonucleotides that contain a U9 or a BrU9 signal, respectively. Both dU5NdU and BrdU5NdU are chimeric RNA/DNA oligonucleotides that contain a U9 or a BrU9 signal, respectively. Both U5NU (22-mer) and mutant-U5NU were previously described.

To evaluate the effect of the different oligonucleotides on transcription termination separate from their effect on transcription initiation, a bead-bound template was employed (25). Initially, selected oligonucleotides were tested for their ability to stimulate premature termination. Ternary complexes containing the radiolabeled G21 transcript were synthesized using the G21(TER29)A78 template, a wild type virus-infected cell extract, ATP, [α-32P]CTP, UTP, and 3′-Ome-GTP. The ternary complexes were isolated, washed, and further incubated with all nucleoside triphosphates in the presence or absence of VTF for the correct recognition of the termination signal by the U5NU recognition factor. Furthermore, the inability of both T5NT and dU5NdU to stimulate premature termination also excludes the possibility that hybrid formation between U5NU and either the nascent transcript or the template strand contributes to the stimulation of premature termination.

A prior report by Shuman and Moss (24) shows that transcription termination was inhibited by incorporation of bromo-UMP into nascent mRNA in vitro, demonstrating that the termination signal was RNA and not DNA. This result also suggested that U5NU recognition factor binding might have been inhibited by substitution of U with BrU. To evaluate the effect of substitution of U with BrU on U5NU stimulation of premature termination, we tested the effect of adding increasing concentrations of a BrdU9-containing chimeric oligonucleotide, BrdU5NdU (Fig. 1), on transcription termination. The addition of BrdU5NdU significantly decreased normal transcription termination, exhibiting a corresponding increase in the read-through product (Fig. 3A). However, BrdU5NdU did not stimulate premature termination. These results demonstrate that the substitution of the U9 signal sequence in the oligonucleotide with BrdU9 interferes with the activity of the termination signal. However, termination was significantly inhibited at a low concentration, exhibiting an \( I_{0.5} \) calculated to be about 30 nM.

The effect of bromouracil substitution of uracil on oligonucleotide stimulation of premature transcription termination was tested directly. A BrU9 containing 36-mer RNA oligonucleotide, pGEM-BrU5NU (Fig. 1), was synthesized by T7 RNA polymerase in vitro. As a positive control, a U9-containing 36-mer RNA oligonucleotide, pGEM-U5NU (Fig. 1), was synthesized as well. Similar to the results obtained with the 22-mer U5NU oligonucleotide, the addition of pGEM-U5NU stimulated premature transcription termination in a concentration-dependent manner (Fig. 3B, lanes 4–9). In contrast, the addition of pGEM-BrU5NU significantly decreased normal transcription termination, exhibiting a corresponding increase in the read-through product, yet failed to stimulate premature transcription termination (Fig. 3C). These results are consistent with the prior observation by Shuman and Moss (24) and demonstrate a requirement for uracil for the oligonucleotide stimulation of premature termination. Lanes 1 and 10 show the unelongated G21 RNA and the 36-mer oligonucleotide, respectively.

To determine whether BrdU5NdU binds to the same site on the recognition factor as U5NU, we tested its ability to act as a competitive inhibitor of U5NU-stimulated premature termination (Fig. 4A). Transcription was carried out in the presence of U5NU and the presence or absence of increasing concentra-
FIG. 2. Oligonucleotide specificity for stimulation of premature early gene transcription termination. Ternary complexes containing the G21 transcript were synthesized using the G21/TER29A78 template, a wild type virus-infected cell extract, ATP, [α-32P]CTP, UTP, and 3'-OMe-GTP. The ternary complexes were isolated, washed, and further incubated in the presence or absence of VTF, which was preincubated in the absence or presence of increasing concentrations of BrdU5NdU (A), pGEM-U5NU (B), or pGEM-BrU5NU (C). The nascent G21 transcript was extended and analyzed as described in Fig. 2. The percent termination or premature termination indicated below the autoradiograph was quantified by scanning the autoradiogram with a densitometer. G21 RNA and the 36-mer pGEM-U5NU (B) or pGEM-BrU5NU (C) were run as size markers. Term, termination product; PT, premature termination products; P, U9 P, pause products.

FIG. 3. Oligonucleotide inhibition of early gene transcription termination. The washed G21 ternary complexes were incubated in the presence or absence of VTF that was preincubated in the absence or presence of increasing concentrations of BrdU5NdU (A), pGEM-U5NU (B), or pGEM-BrU5NU (C). The nascent G21 transcript was extended and analyzed as described in Fig. 2. The percent termination or premature termination indicated below the autoradiograph was quantified by scanning the autoradiogram with a densitometer. G21 RNA and the 36-mer pGEM-U5NU (B) or pGEM-BrU5NU (C) were run as size markers. Term, termination product; PT, premature termination products; P, U9 P, pause products.
The addition of BrdU5NdU prevented premature termination in a concentration-dependent manner (Fig. 4A, lanes 5–9). Furthermore, inhibition of normal termination was observed at high BrdU5NdU concentrations, resulting in increased read-through RNA synthesis. This result demonstrates that both BrdU5NdU and U5NU bind to the same site on the recognition factor. The apparent $K_I$ for BrdU5NdU was calculated to be 37 nM (Table I) from a reciprocal plot of the data in Fig. 4E, similar to the $I_{0.5}$ estimated from the average of multiple repetitions of results represented in Fig. 3A.

To determine whether mutant-U5NU, T5NT, or dU5NdU could bind to the U5NU binding site on the recognition factor, we tested their ability to compete with U5NU. In contrast to BrdU5NdU, neither the mutant-U5NU RNA nor the T5NT ssDNA showed any significant inhibition of the U5NU stimulation of premature termination (Figs. 4, B and C), respectively, demonstrating that neither oligonucleotide binds significantly to the U5NU recognition factor under these conditions. Interestingly, dU5NdU exhibited a moderate inhibition of the U5NU-stimulated premature termination (Fig. 4D). The $K_I$ for dU5NdU was calculated to be 77 nM (Table I). These results demonstrate that dU5NdU has a greater affinity to the recog-

![FIG. 4. Oligonucleotide inhibition of U5NU-stimulated premature termination. The washed G21 ternary complexes were incubated in the absence of any factors (lane 2), the presence of VTF alone (lane 3), VTF plus U5NU (lane 4), and VTF, U5NU, and increasing amounts of the inhibitory oligonucleotides BrdU5NdU (A), mutant-U5NU (B), T5NT (C), or dU5NdU (D) (lanes 5–9). The nascent G21 transcript was extended and analyzed as described in Fig. 2. The percent premature termination, indicated below the autoradiograph, was quantified by scanning the autoradiogram with a densitometer. G21 RNA was run in lanes 1 and 10 as a size marker. E, a graphical representation of the average of multiple analyses is shown. Term, termination product; PT, premature termination products; P, U9 P, pause sites; ●, BrdU5NdU; ○, m-U5NU; ●, T5NT; +, dU5NdU.](image)
TABLE I
Kinetic constants

| Oligonucleotide  | \( A_{0.5} \) | \( K_I \) |
|------------------|----------------|----------------|
| U5NU (9-mer)     | 25             | 0.25           |
| U5NU (13-mer)    | 25             | 0.25           |
| U5NU (17-mer)    | 25             | 0.25           |
| U5NU (22-mer)    | 25             | 0.25           |

BrdU5NdU oligonucleotide (22-mer)

| Oligonucleotide | \( A_{0.5} \) | \( K_I \) |
|------------------|----------------|----------------|
| BrdU5NdU         | 25             | 0.25           |

C50 Extract (NPH I)

| Oligonucleotide | U5NU | NPH I | dATP | VTF | % Release |
|------------------|------|-------|------|-----|-----------|
| USNU             | 25   | 25    | 25   | 25  | 100       |
| NPH I            | 25   | 25    | 25   | 25  | 100       |
| dATP             | 25   | 25    | 25   | 25  | 100       |
| VTF              | 25   | 25    | 25   | 25  | 100       |

FIG. 5. BrdU5NdU oligonucleotide inhibits U5NU-stimulated transcript release. Ternary complexes containing the G21 RNA transcript were synthesized using the G21/TER29/A78 template, a tsC50 mutant virus-infected cell extract (lacking NPH I), ATP, [\( \omega \)-32P]CTP, UTP, and 3′-OMe-CTP. The ternary complexes were isolated, washed, and incubated in the presence of VTF, NPH I, and dATP and the presence or absence of U5NU and increasing concentrations of BrdU5NdU. The bead-bound G21 transcripts (bound (B)) were separated from the released G21 transcripts (free (F)) by magnetic separation and analyzed by electrophoresis through a 15% polyacrylamide gel containing 8% urea. The labeled G21 transcripts were visualized by autoradiography. The percent of RNA released, indicated below the autoradiograph, was quantified by scanning the autoradiograph with a densitometer.

**DISCUSSION**

Early poxvirus genes are unique in that transcription terminates in a signal- and factor-dependent manner (13–15). Effective termination of early gene transcription requires the productive interplay of at least four factors including the virion form of RNA polymerase containing the Rap 94 subunit (16), VTF, a multifunctional transcription factor and mRNA-processing enzyme (14), the ATP-hydrolyzing enzyme NPH I (20, 21), and the signal UUUUUNU in the nascent mRNA (23, 24). The sequence UUUUUNU must be at least 30 nucleotides from the 3′ end of the nascent RNA, indicating that it resides outside of the RNA polymerase (25). This suggests that the UUUUUNU signal is recognized by an undefined essential termination factor, and upon binding this factor initiates (or participates in) the termination/release sequence of events. The function of VTF, a known RNA-binding protein, is undefined, making it a likely candidate for the UUUUUNU recognition factor. It was shown that the D1R subunit of VTF can be cross-linked to bromo-UMP-substituted nascent RNA (31). However, it was not determined whether the protein cross-linked directly to the U5NU signal or to another region in the short transcript. Direct proof of a role of VTF in recognizing the termination signal remains to be obtained.

Recently experiments were conducted in this laboratory in an effort to identify the UUUUUNU binding factor and ascertain its role in termination. These results demonstrated the ability of a U5NU-containing oligonucleotide to stimulate premature termination of early gene transcription irrespective of the template sequence (26). Premature termination stimulation was dependent on Rap 94, VTF, NPH I, and ATP or dATP, demonstrating the involvement of the normal transcription termination machinery (26). Moreover, U5NU was shown to stimulate the release of the G21 transcript from isolated ternary complexes, demonstrating that U5NU-dependent transcript release does not require an extensive interaction with the 5′ tail of the target RNA (26).

In an effort to better understand aspects of the U5NU oligonucleotide-dependent stimulation of premature termination and transcript release, we evaluated the ability of various oligonucleotides to stimulate premature termination in vitro. As shown previously (26), the addition of a 22-mer U5NU-containing oligonucleotide stimulated the premature termination of early gene transcription from the Ter29 template. Importantly, a 22-mer mutant U5NU-containing oligonucleotide failed to do so, demonstrating signal sequence specificity. More-
over, a T5NT-containing ssDNA oligonucleotide was shown to be inactive. In addition, a chimeric RNA/DNA oligonucleotide, dU5NdU, containing a signal consisting of 9 uracil residues linked to 2 deoxyribose, failed to stimulate premature termination. These results demonstrate a requirement for the ribose sugar for the correct recognition of the termination signal by the U5NU recognition factor. Competition studies demonstrated that neither the mutant U5NU RNA nor the T5NT oligonucleotide competed with U5NU for binding to the U5NU recognition factor under these conditions. However, dU5NdU exhibited a moderate ability to inhibit premature termination and, at high concentrations, normal termination. These results show that dU5NdU exhibits a weak affinity to the U5NU recognition factor that is greater than that shown by mutant U5NU and T5NT. Nevertheless, despite its ability to bind weakly to the U5NU recognition factor, dU5NdU is unable to stimulate premature transcription termination.

BrdU5NdU inhibited normal transcription termination, exhibiting a corresponding increase in the read through RNA product. However, it did not stimulate premature termination. Moreover, BrdU5NdU competed with U5NU, demonstrating that both oligonucleotides bind to the same site on the U5NU recognition factor. These results also demonstrate that the presence of the bromine in the 5 position of uracil in BrdU5NdU enhanced its binding activity in comparison to dU5NdU, allowing it to compete with U5NU for binding to the U5NU recognition factor. Nevertheless, despite its ability to bind, BrdU5NdU did not induce the activation of the U5NU recognition factor, resulting in its inability to stimulate premature transcription termination. Furthermore, pGEM-BrU5NU inhibited normal transcription termination yet failed to stimulate premature termination, confirming that 5 bromouracil substitution of uracil prevents the oligonucleotide stimulation of premature termination.

The results presented herein provide experimental support for a model offered by Deng and Shuman (34) (Fig. 7). The U5NU recognition factor exists in two conformations. In the resting state, the U5NU recognition factor is inactive. In the presence of the U5NU signal, the factor binds to U5NU and assumes an active conformation that initiates a sequence of events resulting in the release of the nascent RNA from the ternary complex. Although BrdU5NdU can compete for the binding to the U5NU recognition factor, it fails to induce the required conformational changes that are prerequisite for transcription termination.
to the U5NU recognition factor, both fail to induce its activation. These results demonstrate that oligonucleotide binding alone is not sufficient to induce the active conformation of the U5NU recognition factor. The correct docking of the termination signal to the recognition factor is required for its subsequent activation. This is prevented either by bromine substitution of the 5 position of uracil or by swapping ribose with 2’-deoxyribose. These observations complement prior results reported by Shuman and Moss (24) and Deng and Shuman (34) who showed that substitution of uracil with bromouracil or the presence of a phosphorothioate linkage in the U5NU signal prevented transcription termination. Collectively, these results demonstrate that stimulation of vaccinia virus early gene transcription termination requires a signal that consists of ribouridine nucleosides linked by phosphodiester bonds.

The oligonucleotide length dependence for stimulation of premature termination showed that U5NU RNA as short as 9 bases served as an effective stimulator. In contrast, 7-mers were unable to support premature transcription termination. The addition of two nucleotides to the 7-mer dramatically enhanced the ability of the U5NU signal to activate the recognition factor. The addition of four nucleotides to the 9-mer yielded a 2-fold increase in activity (affinity). However, lengthening to 22 had no additional effect. These data indicate that the oligonucleotide binding site accommodates no more than 13 nucleotides and that an oligonucleotide of 9 nucleotides both binds well and is able to stabilize the active conformation of the recognition factor.

These observations impact directly on the development of oligonucleotide-based anti-poxvirus therapeutic agents. We reason that a short U5NU-containing oligonucleotide might serve as an efficacious anti-poxvirus agent because it would prevent the synthesis of full-sized early mRNA. An inhibitor of early gene expression would be especially valuable because it would not only inhibit virus replication but it would also prevent the synthesis of a family of poxvirus-encoded proteins that inhibit the host response to the poxvirus infection (for review see Ref. 35). Furthermore, inhibition would not be gene-specific, but rather, all RNA polymerases transcribing early genes would be susceptible to U5NU-stimulated premature termination. Experiments are under way to assess the ability of various U5NU-containing oligonucleotides to inhibit transcription within the virion cores.

Finally, U5NU stimulation of premature termination eliminates one model of transcription that would require the transit of the U5NU signal through the RNA polymerase as an obligatory step in termination. Likewise, oligonucleotide stimulation of premature termination in trans does not support the model, which proposes that factor binding to the U5NU signal in the nascent transcript distorts the RNA in the ternary complex such that the growing chain cannot be elongated. Rather, it appears that U5NU binding activates a factor that directly attacks a stable ternary complex (26).

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