Dinucleotide-primed initiation of transcription at specific positions on viral RNA

The priming activities of dinucleotides of all possible base sequences as to the transcription initiation by influenza virus-associated RNA polymerase were investigated. Dinucleotide ApG, complementary to positions 1-2 from the 3' termini of viral RNA segments, was the most active primer and directed the formation of AgGpC; dinucleotide GpC, complementary to positions 2-3, was also an active primer and directed the formation of either GpCpG or GpCpA; but both dinucleotides CpG and CpU, complementary to positions 3-4, were virtually inactive. These results indicate that the transcription is initiated within the first four nucleotides at the 3' termini of viral RNA. Among other dinucleotides, only those hybridizable to viral sequences at their 3'-proximal bases were partially active, implying the essential role of base pairing immediately next to the first phosphodiester bond.

The RNA-dependent RNA polymerase (nucleoside-triphosphate:RNA nucleotidyltransferase (DNA-directed) (EC 2.7.7.5)) associated with the influenza virus plays an essential role in transcription and replication of the viral genome. In vitro studies using this virus-associated RNA polymerase demonstrated that the primary transcription proceeds under such a unique mechanism that the RNA polymerase cleaves the host cell mRNA at specific sites (1, 2) and utilizes the resulting capped RNA fragments as primers for the initiation of viral transcription (3, 4). The capped RNA-dependent transcription initiation can be bypassed if high concentrations of oligonucleotides were added as primers (5-7). The priming activity of oligonucleotides was, however, variable, depending on the sequence and the chain length, suggesting that the transcription initiation takes place at specific sites on viral RNA templates (8). Among dinucleotides examined so far, AG (dinucleoside monophosphates are hereafter represented by two capital letters, e.g. AG represents ApG and GG are known to be good primers for all virus strains (10). Trinucleotide AGC is as active in priming RNA synthesis as the most active dinucleotide, AG (10). All these active oligonucleotides are capable of hybridizing with the 3' ends of the common terminal sequences of 12 nucleotides in length, i.e. either 3'-UCGCCUUUCGUCC or 3'-UCGCUUUCGUCC, present in all the eight RNA segments of the influenza viral genome (9). However, heptanucleotides complementary to the 3'-terminal seven nucleotides within these common sequences, i.e. AGCGAAA or AGCAAAA, are virtually inactive as primers for the transcription initiation (8), suggesting that transcription is initiated over the positions within the seven nucleotides at the 3' termini of viral RNA.

For detailed understanding of the molecular mechanism of primer-dependent initiation of transcription by the influenza virus RNA polymerase, we have performed a systematic and quantitative comparison of the priming activity among dinucleotides of all possible sequences in relation to the start points of transcription on viral RNA. The experimental results show that the dinucleotides-primed transcription is initiated on viral RNAs between positions 2 and 4 from the 3' termini, and the maximum initiation takes place at the third position and in the presence of the AG primer.

Materials and Methods

Results

Oligonucleotide-primed transcription—Transcription by influenza virus-associated RNA polymerase is markedly enhanced by the addition of high concentrations of dinucleotides, which serve as primers for RNA synthesis (5-7). Our previous experiments (8) suggested that, within the two 3'-terminal common sequences of 12 nucleotides in length, the primer-dependent initiation of RNA synthesis takes place within the 3'-proximal seven nucleotides. Detailed analysis showed that trinucleotide AGC is as active as dinucleotide AG, but tetranucleotides AGCA and AGCG show only about 40% the activity of AG (Fig. 1). Heptanucleotides, either AGCGAAA or AGCAAAA, show the lowest priming activity although the stability of primer-template hybrids is the highest. These observations immediately suggest that the transcription is initiated at specific positions near the 3' termini of viral RNAs.

Dinucleotide-primed Initiation of Transcription—The molecular mechanism of primer-dependent RNA synthesis was studied in detail using dinucleotide primers of all possible sequences. For accurate measurement of the priming activity as to transcription initiation, each dinucleoside monophosphate was incubated with the detergent-treated virus in

1 Portions of this paper (including "Materials and Methods," "Discussion," Figs. 3-6, Tables 1 and 2, and "Acknowledgments") are presented in miniprint at the end of this paper. The abbreviations used are: DTT, dithiothreitol; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; BPB, bromphenol blue. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 360 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3796, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
transcribed to RNA by influenza virus RNA polymerase in the presence of a single species of $\alpha^{32P}$-labeled nucleoside 5'-triphosphate. The labeled oligonucleotide products were fractionated by electrophoresis on 30% polyacrylamide gels and measured by counting the radioactivity of gel bands. As summarized in Table 1, the level of $\alpha^{32P}$ incorporation into the oligonucleotide products varied, depending on the combinations of test primers and labeled substrates. This supports the notion that the transcription initiation takes place only at specific sites on the viral RNA templates.

When CTP was added as the sole substrate, it was expected that CMP would be maximally incorporated into the complementary dinucleotide primer AG because C is complementary to G located at the third position from the 3'-termini of template RNAs.

The AG-dependent incorporation of $[\alpha^{32P}]$CTP was the highest among all the reactions examined in this study (Table 1 and Fig. 2). The CG- and UG-dependent incorporation of CTP was 30–40% that of AG, both CG and UG being complementary to the UC sequence only at the 3’-terminal UC sequence and the UC sequence was virtually inactive as a primer for AMP incorporation (initiation at position 1). The observation suggests that the primer activities of dinucleotides also depend on the binding positions relative to viral RNA templates and that the transcription initiation takes place only near the extreme ends of viral RNA and prior to the fourth nucleotide from the 3′-termini. In agreement with this hypothesis, UMP was not incorporated into any of the dinucleotide primers.

Transcription Initiation Positions on Viral RNA—Among the initiation reactions primed by the best-match dinucleotides, the AG-primed incorporation of CMP (initiation at position 3) was about 13-fold higher than the GC-primed incorporation of AMP (initiation at position 4) and about 46-fold higher than the second-position incorporation of AMP (A initiation at position 4) (see Table 1 and Fig. 2A). GC was inactive as a primer for AMP incorporation (initiation at position 5). These observations together suggest that among the three positions, i.e., positions 3–5 from the 3′ termini of viral RNA segments, the transcription initiation was maximum for position 3 (see Fig. 2A). The level was about 10–20% (combined value for GC-dependent incorporation of GMP and AMP) for position 4 and less than 1% for position 5.

In an attempt to determine the transcription initiation activity at position 2, the following two systems were compared: AA- and UA-dependent incorporation of GMP (initiation at position 2) and CG- and UG-dependent incorporation of CMP (initiation at position 3). These four active dinucleotides hybridize only at their 3′ nucleotides with viral RNAs. The level of GMP incorporation was 10–20% the level of...
CMP incorporation (Fig. 2B), suggesting that the level of transcription initiation at position 2 is 10–20% of the level at position 3. This position effect for efficient initiation of transcription is discussed later.

**Priming Activity-Dinucleotide Sequence Relationship**—Up to this point, the priming activities of dinucleoside monophosphates with various sequences were compared at a fixed primer concentration on the initiation of transcription. Fig. 3 shows the results for AG-, GU-, CC-, and AA-dependent CMP incorporation (initiation at position 3), and both the $K_m$ and $V_{max}$ values are summarized in Table 2. The priming activity of AG was saturated above 1 mM, whereas those of GU and CC continued to increase within the range examined, i.e., up to 2 mM for GU and 4 mM for CC. Although the priming activity of AA at 0.24 mM was negligible, it exhibited low activity at 5 mM. Even with saturated concentrations of these noncomplementary dinucleotides, however, the rate of transcription initiation is not as high as that for AG, and the $V_{max}$ values are much lower than that for AG (data not shown).

Fig. 4 shows the effect of variation in the primer concentration for GC-, AA-, AC-, and GU-dependent CMP incorporation (initiation at position 2). The rates of all these reactions increased linearly up to 0.5 mM for the GC- and AA-dependent reactions, 2 mM for the AC-dependent reaction, and 4 mM for the GU-dependent reaction. Again, a small but significant level of GMP incorporation was found for GU at 5 mM, of which the priming activity was negligible at 0.24 mM (see Table 1). These observations together indicate that the priming activity of dinucleotides is correlated with the extent of the pairing with viral RNA: dinucleotides fully hybridizable to viral RNA exhibit the highest activity; dinucleotides which hybridize only at the 3'-proximal bases exhibit intermediate activity; and dinucleotides which hybridize only at the 5'-proximal bases and those showing no complementarity exhibit low activity, if any.

**Sequence of Initiated Oligonucleotides**—The sequences of the initiated oligonucleotides were determined based on the cleavage patterns with various sequence-specific nucleases. For this purpose, the initiated oligonucleotides were separated from labeled substrates by electrophoresis on polyacrylamide gels and isolated by eluting the gel bands. After nuclelease treatment, the cleavage products were identified by electrophoresis on either polyacrylamide gels or DE81 paper.

Fig. 5 shows the electrophoretic patterns on a 20% polyacrylamide gel of the major initiated oligonucleotides. Based on the nuclease sensitivity patterns, the products of the AG- and CG-promoted CMP polymerization reactions were found to be AGC and GCC, respectively. The products on GC-, AC-, and AA-dependent polymerization of GMP were GCC, ACG, and AAG, respectively. AMP was maximally incorporated in the presence of GC. The main product in this reaction was trinucleotide GCA. The sequences were confirmed by electrophoresis on DE81 paper, as shown in Fig. 6. These results together support the above conclusions as to the position effect of transcription initiation on viral RNA templates and the sequence requirement of dinucleotides for the priming activity.

**REFERENCES**


Continued on next page.
Transcription Initiation by Influenza Virus RNA Polymerase

Materials and Methods

Viruses: Influenza virus (A/Hum/SA 09/3) was used throughout this study. The virus was grown in the allantoic sac of 10-day-old embryonated eggs for 48 hr at 37°C. Viruses were precipitated from the allantoic sacs after inverting for 3 hr at 4°C. The precipitated viruses were suspended in 10 mM Tris-HCl (pH 7.4) containing 1 mM MgCl2 and 5% (w/v) glycerol, and stored at -80°C until use.

Chemicals and Reagents: Nucleoside monophosphates were products of Pharmacia. 5'-32P-labeled ribonucleoside triphosphates were purchased from Amersham, while unlabeled ribonucleoside triphosphates were from ICN Biochem and NEN Research. Ribonucleoside ATP was purchased from Pharmacia, while nucleosides A, G, U, and T were products of Yakult Honshu and Daiichi Kagaku, respectively.

Transcription Initiation Assay: The standard reaction mixture contained in 10 ml: 50 mM Tris-HCl (pH 7.4) at 30°C, 5 mM MgCl2, 2 mM GTP, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM G- and U-labeled ribonucleoside 5'-triphosphate, dinitrophenyl ethylenediamine monophosphate and 5 μg of virus. The reaction was carried out for 30 min at 30°C (14). After incubation, 5 ml of an ice-cold sample buffer (7% formamide, 10% glycerol, 0.2% SDS and 0.1% BSA) was added. The mixture was heated for 2 min at 100°C and then directly subjected to polyacrylamide gel electrophoresis. Electrophoresis was carried out in 30% polyacrylamide gel containing 7 M urea. Gels were separated by tray lines and the radioactivity associated with each band was cut out and counted with a liquid scintillation spectrometer.

Results

Transcription by influenza virus-associated RNA polymerase is initiated near the 5' end of viral RNA segments. This notion is based on the following observations: (i) all the viral RNA segments carry one or more complementary sequences, in three' terminal viral RNA sequences, are active in priming transcription but tetranucleotides are less active, and hepatitis C virus RNA polymerase, which are believed to be the targets for transcription by a single species of the core RNA polymerase, the RNA viruses in the presence of either oligonucleotides or capped RNA primers are as large as viral RNA segments; (ii) both A and G, that are complementary to the 3' terminal viral RNA sequences, are active in priming transcription but tetranucleotides are less active, and hepatitis C virus RNA polymerase, which are believed to be the targets for transcription by a single species of the core RNA polymerase, the RNA viruses in the presence of either oligonucleotides or capped RNA primers are as large as viral RNA segments; (iii) both A and G, that are complementary to the 3' terminal viral RNA sequences, are active in priming transcription but tetranucleotides are less active, and hepatitis C virus RNA polymerase, which are believed to be the targets for transcription by a single species of the core RNA polymerase, the RNA viruses in the presence of either oligonucleotides or capped RNA primers are as large as viral RNA segments; (iv) transcripts primed by dinucleotides carry the sequence complementarity to the 3' terminal sequences of viral RNA segments, and v) transcripts primed by capped RNA also carry the sequence complementarity to the 3' terminal sequences of viral RNA segments, and vi) there is a possible chance that the initiation position(s) using dinucleotide/primer trinucleotide synthesis and to determine the structural requirements for the primer molecules.

Discussion

At the beginning of this study, we predicted the transcription start positions on the basis of the combinations of dinucleotide primers and substrate. The example, 6 might be transcribed at only position 3 within the 3' terminal sequences of seven nucleotides in length 6 might be transcribed at either position 3 or 4, and it might be transcribed at positions 1, 4, 5, and 6. With this to the primers, we predicted that dinucleotides fully hybridized to viral RNA would show the greatest priming ability, for example, 6 might be the best primer for transcription initiation at position 3, either C or G might be the best primer for position 4, and A might be the best primer for positions 5 and 6. With the experimental results, the initiation position is determined from double reciprocal replots. The experimental results clearly indicated that the level of transcription initiation is determined by the combination of the start positions relative to viral RNA template and the complementarity of dinucleotides to the viral RNA segments. The initiation position(s) using dinucleotide/primer trinucleotide synthesis and to determine the structural requirements for the primer molecules.

The dinucleotide-initiated transcription was maximum at position 3 from the 3' terminal of viral RNA segments. Both positions 2 and 4 were also active, but the transcription levels were 50-60 % of the level for position 3. McGeoch and Strobel (31) observed that guanosine alone is not sufficient to stimulate the synthesis of RNA with 5' G sequence, indicating that the initiation at position 3 is important since guanosine bound to C residue at position 4 might be transferred to RNA with 5' GG sequence.

With respect to overall RNA synthesis, however, position 4 is equally active if one uses trinucleotide AAG (8), presumably because the two-dimensional binding is stronger for trinucleotide than dinucleotide. On the other hand, capped RNA-dependent transcription was maximally initiated at position 2 (13). GMP was the only substrate incorporated to capped RNA fragments generated by the RNA polymerase-associated endonuclease (12). The transcription initiation also takes place at position 3, although it is undetectable in other positions including positions 1 and 4. Thus, the transcription initiation position is higher in the RNA polymerase than capped RNA primers, suggesting that oligonucleotide primers can reach the enzyme catalytic center of the first phosphodiester bond more easily than capped RNA, which is fixed to the enzyme at both the catalytic center and the capping site.

The position effect for transcription initiation over viral RNA sequences is in good agreement with our previous observation that influenza virus-associated RNA polymerase is bound to the 3' unidentified position of viral RNA and is not dissociated even an equilibrium centrifugation in cesium chloride (14) and cesium trifluoroacetate (15). The primer activity of dinucleotides provides some new opportunities for investigation of the topology of the RNA polymerase on viral RNA. The position effect might also be due to the fact that RNA enzymes are not associated with the high-molecular weight the polyribosomes. And only four bases from the 3' end of the template are exposed to the primer.

For maximal priming of the transcription initiation, dinucleotides should be complementary to viral RNA sequences immediately upstream from the initiation position. Among the two species of dinucleotides complementary to these viral RNA sequences at only single bases, those hybridizable at their 3' bases were active, but those hybridizable at their 5' bases were virtually inactive. These observations indicate that the base pairing immediately next to the initiation position plays an important role in the priming activity. Essentially the same requirement was found for oligonucleotides primers in the RNA-dependent RNA synthesis by G poler RNA polymerase (16). The primer activity of dinucleotides whose bases are hybridizable with the template was approximately twice that of dinucleotides which are hybridizable only at their 3' terminal single bases. In the case of RNA-dependent DNA synthesis, the primer activity of complementary oligonucleotides becomes maximum at the primer chain length of 4 for GC pairing and of 6 for AT pairing (17). The presence of unpaired bases upstream from these regions does not interfere with the priming activity.

Acknowledgments: We thank A. Kato, K. Kayata and R. Fukuda for the preparation of the virus and discussion.

Table 1.

<table>
<thead>
<tr>
<th>Primer activity of various dinucleotides</th>
<th>Initiation position</th>
<th>Template length</th>
<th>Initiation (f mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>140</td>
</tr>
<tr>
<td>3</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>2,500</td>
</tr>
<tr>
<td>4</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>1,400</td>
</tr>
<tr>
<td>5</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>980</td>
</tr>
<tr>
<td>6</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>960</td>
</tr>
<tr>
<td>7</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>6.5</td>
</tr>
<tr>
<td>9</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th>Primer activity of various dinucleotides</th>
<th>Initiation product</th>
<th>Template length</th>
<th>KM (μM)</th>
<th>Vmax (f mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>0.2</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>0.25</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>GMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>CMP</td>
<td>32P-labeled ribonucleoside 3'-triphosphate</td>
<td>6.5</td>
<td>20</td>
</tr>
</tbody>
</table>

The initiation reaction was carried out under the standard conditions in the presence of a 4-dinucleotide primer at 24 μM. Nucleotides complementary to the viral RNA templates are underlined. Both the KM and Vmax values were determined from double reciprocal replots.
Transcription Initiation by Influenza Virus RNA Polymerase

Fig. 3. Effect of the dinucleotide concentration on the priming activity as to transcription initiation. Formation of tri- and tetranucleotides was measured in the presence of various concentrations of the indicated dinucleotides and [α-32P]TP. The scales for GU, CC and AA are expanded in B.

Fig. 4. Effect of the dinucleotide concentration on the priming activity as to transcription initiation. Formation of tri- and tetranucleotides was measured in the presence of the indicated dinucleotides and [α-32P]TP.

Fig. 5. Sequence analysis of tri- and tetranucleotides. The isolated tri- and tetranucleotides were treated with various nucleases, and the products were analyzed by electrophoresis on 20% polyacrylamide gel, in which dephosphorylated oligonucleotides migrate in the order of chain length. A1, untreated AGC; 2, RNase T1; 3, RNase T2. B1, untreated AGC; 2, RNase T1; 3, RNase T2; 4, nuclease P1. C1, untreated AGC; 2, RNase T1; 3, RNase T2; 4, nuclease P1.

Fig. 6. Sequence analysis of tri- and tetranucleotides. The isolated tri- and tetranucleotides were treated with various nucleases, and the products were analyzed by electrophoresis on DE81 paper. A1, untreated AGC; 2, RNase T1-treated; 3, RNase T2-treated. B1, untreated AGC; 2, RNase T1; 3, RNase T2; 4, nuclease P1. C1, untreated AGC; 2, RNase T1; 3, RNase T2; 4, nuclease P1.