RNA Polymerase of Influenza Virus

DINUCLEOTIDE-PRIMED INITIATION OF TRANSCRIPTION AT SPECIFIC POSITIONS ON VIRAL RNA*

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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 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 2.7.7.6)) 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′-UCGCUUUUUCGUC or 3′-UCGUUUUUCGUC, 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 dinucleotide-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 the

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Fig. 1. Effect of the oligonucleotide chain length on the priming activity of influenza viral transcription. RNA synthesis was carried out for 30 min at 30 °C under the standard reaction conditions in the presence of one of the indicated oligonucleotides at 0.24 mM. The activities are expressed as values relative to that for AG, which showed the incorporation of about 1 nmol of [α-32P]UTP.

Fig. 2. Effect of the dinucleotide sequence on the priming activity as to transcription initiation. Formation of trinucleotides was measured under the standard reaction conditions in the presence of one of the indicated dinucleotides at 0.24 mM and α-32P-labeled ribonucleoside 5'-triphosphate. The amounts of trinucleotides are expressed as values relative to that for AGC, which was 2.5 nmol on the 30-min reaction (see Table 1). The bases with stippling are complementary to the template RNAs, while X represents a noncomplementary base. A, dinucleotides fully complementary to viral RNA; B, dinucleotides complementary to viral RNA only at their 3'-terminal bases.

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presence of a single species of α-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 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 [α-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 3'-terminal UC sequence only at the 3'-terminal G residue, suggesting that the primer activities of dinucleotides depend on the complementarity to viral RNA templates. GG exhibited an intermediate activity because of the terminal G residue, suggesting that the primer activities of AG, AA, and UA, both being complementary at their 3' termini, were virtually inactive or showed less than 1%, if any, the activity of AG.

Guanine nucleotide could be transcribed from viral RNAs at positions 2 and 4 of the 3'-terminal sequences, and the active primers might be NA (N represents A, G, C, or U) and GC, respectively. The experimental results indicate that CMP incorporation is high for the three species of dinucleotides, GC, AA, and UA (see Table 1 and Fig. 2). Sequence analysis indicated that GC directed the formation of GCG (for details, see Figs. 5 and 6), which is fully complementary to viral RNAs between positions 2 and 4. The other three dinucleotides with the C residue at their 3' ends, i.e., AC, CC, and UC, exhibited 10-20% of the activity of GC. Again, these low levels of activity might be due to low levels of primer template pairing. All the dinucleotides lacking the complementarity to template RNA at the 3' bases of primers were virtually inactive. Accordingly, AA and UA, both being complementary at their 3' termini with the 3'-terminal U residue of all eight viral RNA segments, directed the formation of AAG and UAG by transcribing the C residue at the second position.

Adenine nucleotide is complementary to positions 1 and 4-7 of viral RNA. Among the dinucleotides fully hybridizable to viral RNA at sites prior to each of the template U residues, GC was the only active acceptor for AMP incorporation (see Fig. 2), all the others including CG, which is complementary to the GC sequence located between the third and fourth nucleotides from the 3' termini, being virtually inactive as primers (Table 1). This 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 GMP (initiation at position 4) and about 46-fold higher than the second position. 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

Transcription Initiation Positions on Viral RNA
Primed Activity-Dinucleotide Sequence Relationship—Up to this point, the priming activities of dinucleoside monophosphates with various sequences were compared at a fixed concentration, i.e. 0.24 mM, which is higher than the \(K_m\) value (0.05–0.10 mM) for ApG-primed initiation. It is, however, expected that the priming activities of the dinucleotides with low affinity to the templates might be increased by adding higher concentrations of the respective dinucleotides. To test this possibility, we examined the effect of variation in the 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 incorporation of GMP (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 nuclease 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-primed CMP polymerization reactions were found to be AGC and CGC, respectively. The products on GC-, AC-, and AA-dependent polymerization of GMP were GCG, ACG, and AAC, 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.

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Materials and Methods

Viruses: Influenza virus A/PR/8/34 (H1N1) was used throughout this study. The virus was grown in the allantoic sacs of 10-day-old embryonated eggs for 48 hr at 37°C. Virions were precipitated from the allantoic sacs after mixing with 3 ml of 0.125 M NaCl, 0.03 M Tris-KCl (pH 8.0) at 3°C and then purified by centrifugation through a sucrose step gradient (14). The purified virions were suspended in 10 mM Tris-KCl (pH 7.6) containing 1 mM DTT and 100 µg/ml glyceral, and stored at –80°C until use.

Chemicals and Reagents: Dinucleoside monophosphates were products of Pharmacia. 5'- and 3'-32P-labeled ribonucleoside 5'-triphosphates were purchased from Amerham, while unlabeled ribonucleoside 5'-triphosphates were from Fluka Biochemica and Boehringer. Ribonucleotides T1 and U2 were purchased from Pharmacia, while nucleotides A3 and U3 were products of Yaman Sho and Dai-ichi Kagaku Kogyo respectively.

Transcription Initiation Assay: The standard reaction mixture contained in 10 ml: 50 mM Tris-KCl (pH 7.5), 0.5 mM MgCl2, 2 mM DTT, 100 µM NaCl, 0.1% NP-40, 0.1 mM 5'-32P-labeled ribonucleoside 5'-triphosphate, dinucleoside monophosphate and 5 µg of virions. The reaction was initiated by mixing the virions with 3 µl of primer in 10 mM Tris-KCl (pH 7.6) containing 1 mM DTT and 100 µg/ml glyceral, and stored at –80°C until use.

Proteins: Maximal priming of the transcription initiation, dinucleotide primers 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 single base, those hybridizable at their 5' bases are active, but those hybridizable at their 3' bases are 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 oligonucleotide primers in the RNA-Dependent RNA synthesis by random RNA polymerase (16). The priming 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 RNA synthesis, the priming activity of complementary oligonucleotide becomes maximum at the primer chain lengths 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, H. Nagata and H. Fukuda for the preparation of the virus and discussion.

Table 1.

| Position | Labelled Substrate | Dinucleotide Primer | Incorporation (fmol) |
|----------|--------------------|---------------------|---------------------|
| 2        | GTP                | AG                 | 150                |
| 3        | CTP                | AG                 | 160                |
| 4        | GTP                | GC                 | 70                 |
| 5        | ATP                | GC                 | 25                 |
| 6        | ATP                | GC                 | 15                 |

Table 2.

| Position | Labelled Substrate | Dinucleotide Primer | Incorporation (fmol) |
|----------|--------------------|---------------------|---------------------|
| 2        | GTP                | AG                 | 0.2                |
| 3        | CTP                | AG                 | 0.05               |
| 4        | GTP                | GC                 | 0.3                |

The initiation reaction was carried out under the standard conditions in the presence of a 4-dinucleoside primer at 0.44 µM. Nucleotides complementary to the viral RNA templates are underlined.

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 tightly bound to a unidentified position of viral RNA and is not dissociated even an equilibrium centrifugation in cesium chloride (14) or cesium trifluoromethane (15). The primer activity of dinucleotides provides some new possibilities for investigation of the topography of the RNA polymerases on viral RNA. The position effect might also be due to the fact that RNA templates are associated with the nucleocapsid and the polymerase proteins and only four bases from the 3'-end of the template are exposed to the primers.

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Fig. 3. Effect of the dinucleotide concentration on the priming activity as to transcription initiation. Formation of trinucleotides was measured in the presence of various concentrations of the indicated dinucleotides and [α-32P]CTP. 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 trinucleotides was measured in the presence of the indicated dinucleotides and [α-32P]UTP.

Fig. 5. Sequence analysis of trinucleotides. The isolated trinucleotides 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. (A) 1, untreated AGC; 2, RNase T1-treated; 3, RNAse T2; 4, nuclease P1. (B) 1, GGC; 2, RNase T1; 3, RNase T2; 4, nuclease P1.

Fig. 6. Sequence analysis of trinucleotides. The isolated trinucleotides were treated with various nucleases, and the products were analyzed by electrophoresis on DE81 paper. (A) 1, untreated AGC; 2, RNase T1-treated; 3, RNase T2-treated. (B) 1, GGC; 2, RNase T1; 3, nuclease P1. (C) 1, untreated GGC; 2, RNase T1; 3, nuclease P1.