RNA and DNA Hydrolysis Are Catalyzed by the Influenza Virus Endonuclease*

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The influenza virus polymerase complex contains a metal ion-dependent endonuclease activity, which generates short capped RNA primer molecules from capped RNA precursors. Previous studies have provided evidence for a two-metal ion mechanism of RNA cleavage, and the data are consistent with a direct interaction of a divalent metal ion with the catalytic water molecule. To refine the model of this active site, we have generated a series of DNA, RNA, and DNA-RNA chimeric molecules to study the role of the 2'-hydroxy groups on nucleic acid substrates of the endonuclease. We could observe specific cleavage of nucleic acid substrates devoid of any 2'-hydroxy groups if they contained a cap structure (m7GpppG) at the 5'-end. The capped DNA endonuclease products were functional as primers for transcription initiation by the influenza virus polymerase. The apparent cleavage rates were about 5 times lower with capped DNA substrates as compared with capped RNA substrates. Cleavage rates with DNA substrates could be increased to RNA levels by substituting the deoxyribose moieties immediately 5' and 3' of the cleavage site with ribose moieties. Similarly, cleavage rates of RNA substrates could be lowered to DNA levels by exchanging the same two ribose groups with deoxyribose groups at the cleavage site. These results demonstrate that the 2'-hydroxy groups are not essential for binding and cleavage of nucleic acids by the influenza virus endonuclease, but small differences of the nucleic acid conformation in the endonuclease active site can influence the overall rate of hydrolysis. The observed relative cleavage rates with DNA and RNA substrates argue against a direct interaction of a catalytic metal ion with a 2'-hydroxy group in the endonuclease active site.

The influenza virus contains a negative strand RNA genome consisting of eight RNA segments encoding a total of 10 viral proteins. The vRNA* segments are transcribed and replicated by a virus-encoded polymerase complex. The polymerase complex also contains an endonuclease activity, which is required prior to transcription initiation for the generation of short, capped RNA primer molecules. Nuclear host pre-mRNAs are the most likely endonuclease substrates for the generation of primer molecules in infected cells (1). The influenza virus polymerase is a trimer of the subunits PA, PB1, and PB2. Prior to transcription initiation, the trimeric polymerase complex is bound to both ends of a nucleoprotein-coated single-stranded RNA genome segment forming a noncovalent circular structure, the viral ribonucleoprotein (RNP) (2–4). Separate binding sites for both the 5'- and the 3'-end of the vRNA have recently been mapped on the polymerase subunit PB1 (5, 6). The binding of the polymerase complex to both vRNA ends has previously been found to be a prerequisite for the activation of endonuclease and transcription initiation activities (5, 7, 8).

The process of viral transcription in the infected cell is catalyzed by the RNP-associated polymerase complex. Transcription starts with the binding of capped host pre-mRNAs, which are subsequently cleaved by the endonuclease at distinct sequence-dependent positions 9–15 nucleotides downstream of the cap structure (9–12). The resulting capped RNA oligonucleotides contain 3'-hydroxyl groups. They are used as primers for the initiation of transcription (7, 10). The location of the endonuclease active site on the polymerase complex is still unknown. Although it has been reported that some polymerase active sites of RNA polymerases could catalyze nuclease reactions (13, 14), there is strong evidence to suggest the presence of a separate endonuclease active site on the influenza virus polymerase complex. Inhibitors selective for either endonuclease or polymerase activities are known (15, 16), and endonuclease and polymerase activities show distinct differences in metal ion preference (17). The endonuclease active site may in fact be located on a different polymerase subunit, because specific PB2-directed antibodies selectively abolish the endonuclease activity in vitro (18, 19), whereas the polymerase active site has been mapped to the PB1 subunit (20, 21).

The cap-dependent endonuclease activity may be regarded as a rather unique enzyme activity of influenza viruses. However, for the design and development of selective inhibitors of this activity, it will be important to identify the most closely related cellular enzymes and to get a better idea of the endonuclease active site architecture. Recent experiments have suggested a two-metal ion mechanism of RNA cleavage for the influenza virus endonuclease reaction based on cooperative binding of metal ions and synergistic activation by metal ion combinations as compared with single divalent metal ion reactions (17). Among the group of metalloproteins for which similar catalytic mechanisms have been proposed, there are examples of both DNA and RNA hydrolases (22, 23). Direct discriminatory interactions with the 2'-hydroxy groups of RNAs seems to be rare in this group of enzymes. This is in contrast to the metal-independent RNases like RNase A and RNase T1, which directly interact with 2'-hydroxyl groups to distinguish RNA from DNA molecules. They also use the 2'-hydroxy groups as nucleophiles in the forward reaction to generate cyclic phosphates. In the case of RNA hydrolysis by the ribozyme RNase P, evidence has been presented for an inner sphere water interaction of a divalent metal ion with the 2'-hydroxy group at the cleavage site, which appeared to be a major determinant of RNA specificity of cleavage (24, 25). How-

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* The abbreviations used are: RNP, ribonucleoprotein; vRNA, viral genomic RNA; G20, cap 1-(2'-methoxy)-Gem20 RNA; dG20, cap 1-(2'-methoxy)-Gem20 DNA.
ever, most metal-dependent nuclease proteins can use both RNA and DNA as substrates, or RNA-DNA discrimination is achieved by nucleic acid conformational recognition elements rather than by direct interaction or active exclusion of the 2'-hydroxy group.

Our understanding of the rules that govern cleavage site and substrate selectivity of the influenza virus endonuclease is still very limited. Here, we used a panel of DNA, RNA, and DNA-RNA chimeric oligonucleotides to define the role of 2'-hydroxy groups on the influenza virus endonuclease substrates. Although the endonuclease presumably is specific for RNA substrates in vivo, we observed efficient cleavage of DNA oligonucleotides in vitro albeit with a 5-fold reduced cleavage rate as compared with RNA. The cleavage under physiological buffer conditions required the presence of a cap structure on the DNA or RNA substrates. It therefore appears that the specificity for RNA cleavage by the influenza virus endonuclease in vivo is mainly determined by the specific interaction of the polymerase complex with capped RNA and the absence of capped DNA in the cell. Two nucleotides in the endonuclease active site also contribute to RNA discrimination. On DNA substrates, the substitution of the deoxyribose moieties immediately 5' and 3' of the cleavage site with ribose moieties resulted in nucleic acid substrates that were cleaved with rates identical to that of RNA.

**EXPERIMENTAL PROCEDURES**

**Materials—**Influenza virus A/FPR/8/34 RNP's were prepared from purified influenza virus particles on glycerol gradients as described (4). RNPs concentration was determined from the analysis of genomic RNA content after phenol extraction (17). RNases for RNA sequencing and unlabeled ribonucleoside 5'-triphosphates were purchased from Amersham Pharmacia Biotech; S-adenosyl-l-methionine was purchased from Sigma; radiolabeled ribonucleotide 5'-triphosphates (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech; and vaccinia virus guanlyltransferase was purchased from Life Technologies, Inc. RNase inhibitor was obtained from Roche Molecular Biochemicals.

**Nucleic Acid Synthesis—**The endonuclease substrate nucleic acids were based on the sequence of Gem20 RNA, 5'-GAUAUCUCAAGCUUUGCAUC-3' (17). The DNA oligonucleotides used in this study always contain thymidine nucleotides at the uridine positions of the RNA. The chimeric oligonucleotides were named according to the parent molecule, G20 for Gem20 RNA or dG20 for Gem20 DNA, with an added number for the position of the substitution and a suffix, R or D, depending on whether the substitution was a ribo- or deoxyribonucleotide. For example, G20-11D was a capped Gem20 RNA molecule containing a deoxyribonucleotide at position 11, and dG20-11,12R was a capped Gem20 DNA molecule containing ribonucleotides at positions 11 and 12. All oligonucleotides were chemically synthesized, phosphorylated, and enzymatically capped using vaccinia virus capping enzyme following published procedures (26–28). The oligonucleotides were purified by polyacrylamide gel electrophoresis on 15% gels containing 8 M urea and quantified according to the amount of radioactive GTP incorporated into the cap structure.

**Endonuclease and Transcription Initiation Reactions—**Except when indicated so in the figure legends, endonuclease reactions were performed in 5-μl reaction mixtures containing 1 nM RNP, 50 mM Tris-HCl, pH 8, 100 mM KCl, 0.5 units/μl RNasin, 0.25 μg/μl bovine serum albumin, 0.3% Triton X-100, 0.015–0.15 μM [γ-32P]cap-labeled nucleic acid substrate. After incubation at 31 °C for the indicated times, the reactions were stopped by the addition of 5 μl of loading buffer (0.5 mM EDTA, 90% formamide, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol). The samples were then incubated for 2 min at 98 °C and loaded onto 20% acrylamide sequencing gels containing 7 M urea. Band intensities were quantified with a Storm PhosphorImager (Molecular Dynamics, Inc.) using ImageQuant version 4.2a software. For transcription initiation reactions, 10 μM CTP-Mg was added to the samples prior to the RNPs under the same reaction and incubation conditions as described above. Nucleic acid cleavage activity (A) was expressed as relative product formation from the PhosphorImager band volumes according to the equation A = (P)/(P + S) where P represents product and S represents substrate. Time response curves were fitted to the exponential equation A = A_0 (1 - e^(-kapp t)) to obtain the pseudo-first-order kinetic constants k_0 and k_∞ of the cleavage reactions. Transcription initiation reactions from 11-mer primers and coupled endonuclease/transcription initiation reactions from 20-mer substrates were run under conditions where about 50% of the substrates were cleaved and elongated. In that case, K_0(0) and k_∞ were calculated from fitting hyperbolic standard curves to the data sets using the equation A = (k_0 + S)/(K_0 + S), where S represents the concentration of CTP in the transcription initiation reaction.

**RESULTS**

**DNA Oligonucleotides Are Specifically Cleaved and Used for Transcription Initiation by the Influenza Virus Polymerase Complex—**To study the influenza virus endonuclease reaction we used a previously characterized model substrate of 20 nucleotides in length, Gem20-M RNA (20). This RNA substrate contains a cap I structure and is specifically cleaved by the endonuclease activity of the polymerase complex at a distinct site to generate a capped 11-mer oligonucleotide, G11, as determined by direct RNA sequencing (17). This cleavage was dependent on a methylated cap structure on the RNA substrates similar to what has been established before for other RNA substrate sequences (10). Surprisingly, a capped 20-mer DNA oligonucleotide of identical sequence (dG20) was also found to be specifically cleaved at a single site (Fig. 1a). The cleavage product co-migrated with a capped 11-mer DNA marker (dG11). DNA cleavage was dependent on the presence

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2 K. Klumpp and L. Doan, unpublished results.
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The elongation in the presence of CTP (Fig. 1b) was also influenza endonuclease-specific as demonstrated by 11R; c, RNA (G20) molecules were incubated with 1 nM RNP under the same conditions as described in the legend to Fig. 1 for 60, 30, and 15 min at 31 °C, respectively, to achieve about 50% conversion of substrate. CTP concentrations varied between 0.1 nM and 10 μM; a, lane 1, MgCl2 omitted; lane 2, CTP omitted; lanes 3–8, endonuclease reactions in the presence of 0.001, 0.01, 0.1, 1, 10, and 100 μM CTP. c, lane 1, MgCl2 omitted; lanes 2–11, 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 10 μM CTP. d, the relative amount of transcription initiation product was determined and plotted against the CTP concentration. Black squares, G20; white circles, dG20–11R; black triangles, dG20. The DNA showed the slowest rate and highest Km value. The chimeric molecule showed an intermediate rate and a Km value similar to the one obtained with the RNA substrate. e, schematic representation of the sequences of vRNA template as present on influenza virus RNP and the G11 (or dG11) primer molecules aligned for transcription initiation in the presence of CTP (see also Ref. 17).

Fig. 2. CTP dependence of the combined endonuclease transcription initiation reaction. a, 20-mer DNA (dG20); b, chimeric (dG20–11R); c, RNA (G20) molecules were incubated with 1 nM RNP under the same conditions as described in the legend to Fig. 1 for 60, 30, and 15 min at 31 °C, respectively, to achieve about 50% conversion of substrate. CTP concentrations varied between 0.1 nM and 10 μM. a, lane 1, MgCl2 omitted; lane 2, CTP omitted; lanes 3–8, endonuclease reactions in the presence of 0.001, 0.01, 0.1, 1, 10, and 100 μM CTP. b, lanes 1–7, 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 10 μM CTP. d, the relative amount of transcription initiation product was determined and plotted against the CTP concentration. Black squares, G20; white circles, dG20–11R; black triangles, dG20. The DNA showed the slowest rate and highest Km value. The chimeric molecule showed an intermediate rate and a Km value similar to the one obtained with the RNA substrate. e, schematic representation of the sequences of vRNA template as present on influenza virus RNP and the G11 (or dG11) primer molecules aligned for transcription initiation in the presence of CTP (see also Ref. 17).
TABLE I
Transcription initiation with CTP from model substrates

| Substrate       | $K_m$ (app)$^a$ [nM] | $k_{app}$ [min$^{-1}$] | $k_{app}/K_m$ (app) [×10$^6$] |
|-----------------|-----------------------|-------------------------|-------------------------------|
| G20             | 10 ± 2                | 0.05 ± 0.01             | 8.3                           |
| dG20            | 83 ± 11               | 0.009 ± 0.002           | 2.8                           |
| dG20–11R        | 15 ± 7                | 0.025 ± 0.009           | 2.5                           |
| G11             | 12 ± 3                | 0.057 ± 0.009           | 6.1                           |
| dG11            | 143 ± 15              | 0.05 ± 0.01             | 2.5                           |
| dG11–11R        | 4 ± 1.5               | 0.08 ± 0.01             | 1.5                           |

$^a$ $K_m$ (app) for CTP as determined from fitting of a hyperbolic equation to data from experiments as shown in Figs. 2 and 3. Average values and S.D. values shown are derived from three independent experiments.

$^b$ $k_{app}$ determined as the rate of the formation of (d)G11 + 1 nucleotide in the single turnover reaction.

Fig. 3. CTP dependence of transcription initiation from DNA and RNA primer molecules. Capped primer nucleic acids as indicated above the gels were incubated with 1 nM RNP under the same conditions as described in the legend to Fig. 1 for 10 min at 31 °C. CTP concentrations varied between 0.2 nM and 2 μM. The migration positions of the substrates and transcription initiation products on a denaturing acrylamide gel are indicated on the left. DNA oligonucleotides were migrating faster than RNA oligonucleotides of the same size and sequence. The plot shows a PhosphorImager analysis of the relative product formation as apparent on the gels. Whereas the apparent initiation rates were similar, the $K_m$ values varied over a 35-fold window (see Table I). White circles, dG11–11R; black squares, G11; black triangles, dG11.

were similar to those observed in the coupled endonuclease/ transcription initiation reaction. Again, $K_m$ (app) for CTP was significantly higher for transcription initiation from the DNA primer dG11 as compared with initiation from the RNA primer G11. Surprisingly, replacing the deoxyribonucleotide at position 11 of dG11 with a ribonucleotide generated a primer (dG11–11R) that was initiated with an apparent $K_m$ even lower than that observed with G11 RNA. The apparent rates of transcription initiation were virtually identical with any of the three 11-mer substrates under these conditions.

These results showed that 2'-hydroxy groups on the nucleic acid substrates were not required for catalysis by neither the endonuclease nor the polymerase active sites. However, subtle differences in conformation of the nucleic acid 3'-end at the polymerase active site could influence the efficiency of transcription initiation.

Cleavage Specificity of dG20 DNA at Position 11 Is Maintained Even after Introduction of Ribosyl Moieties at Different Positions of the Sequence—Cleavage of different species of capped RNA molecules by the influenza virus endonuclease has been observed before at varying positions in a region between 9 and 15 nucleotides downstream of the cap structure depending on the RNA sequence used in the cleavage reaction. At present, the factors involved in the influence of the sequence environment around the cleavage site on cleavage site selection and cleavage efficiency are not very well understood (9–12, 29, 30). Previously, Olsen et al. (27) have introduced a deoxyribosyl moiety at the cleavage site of a different RNA oligonucleotide substrate of the influenza virus endonuclease. This modification induced a one-nucleotide downstream shift of the cleavage site from the preferred position 13 to position 14 on an avian myeloblastosis virus RNA 4-derived sequence (27). These observations suggested a considerable flexibility in the distance between cap-binding and endonuclease sites on the polymerase complex and a significant preference for cleavage at ribosyl moieties on the nucleic acid substrate. To further analyze the mechanism of cleavage site choice, we therefore devised a series of DNA oligonucleotides based on the dG20 sequence containing single ribosyl moieties in the region of nucleotides 8–12.

Surprisingly, the endonuclease did not follow the ribonucleotide positions on the DNA substrates. Instead, the specific endonuclease cleavage site at position 11 was preserved as the major cleavage site in all dG20-derived oligonucleotides independently of the position of the ribosyl moiety (Fig. 4). With these DNA substrates, the endonuclease showed slight differences in cleavage rates and in the efficiency of using a second specific cleavage site at position dG16 (Fig. 4, star). However, the major cleavage site was at dG11 in all cases. Only the substrate dG20–10R also showed a low level of cleavage at the ribosyl position 10 in addition to predominant cleavage at deoxyribonucleotide position 11. These results suggested that in the case of the dG20 RNA sequence the chimeric nucleic acid substrates were bound in a single, fixed position relative to cap-binding and endonuclease sites.

Endonuclease Cleavage Rates Are Regulated by Nucleotides 11 and 12 on G20-derived Substrates—As shown above with the dG20-derived nucleic acid sequence, cleavage site choice at position 11 was dominant even when cleavage had to occur at a deoxyribosyl group and even if ribonucleotides were present in the previously reported endonuclease range of 9–15 nucleotides from the cap structure. These results demonstrated that both DNA and RNA molecules were surprisingly efficient endonuclease substrates. There were, however, measurable differences in cleavage rates when RNA and DNA substrates were compared in this assay. As shown in Fig. 5 (and Table II), G20 RNA was cleaved about 5 times faster than dG20 DNA by the influenza virus endonuclease.

To investigate the nucleotide positions involved in this low
but significant level of RNA-DNA discrimination, we measured the cleavage rates with dG20 derivatives containing single ribonucleotide along the sequence. The molecules with ribonucleotide replacements at positions between the cap structure and position 10 were all cleaved most efficiently at deoxyribonucleotide dG11 with cleavage rates that were indistinguishable from those of dG20 DNA, as exemplified by dG20–8R and dG20–10R (Table II). Substitution of deoxyribonucleotide 11 with a ribonucleotide led to a 3-fold increase in cleavage rate with dG20–11R. Introducing a ribonucleotide at position 12 also showed a low but reproducible improvement in endonuclease activity (dG20–12R; Fig. 5). The combination of ribonucleotide replacements at positions 11 and 12 generated a nucleic acid substrate, dG20–11,12R, that was cleaved as fast as G20 RNA (Fig. 5). To independently assess the relative cleavage rate with a different RNA sequence, we measured endonuclease activity with RNA oligonucleotide “7A16A,” which is a G20 RNA derivative with a double mutation of U7A and G16A. This RNA molecule was cleaved with a rate indistinguishable from that of G20 RNA (Fig. 5, Table II). As mentioned above, the RNA molecules migrated more slowly than the DNA oligonucleotides on the acrylamide gels (Fig. 5).

These experiments showed that three related oligonucleotides, two RNA molecules and one DNA molecule with two ribonucleotide replacements at positions 11 and 12, were all cleaved with identical, high rates by the influenza virus endonuclease. If these two positions in the G20/dG20 sequence indeed determined the RNA/DNA-like rates of cleavage, then single deoxynucleotide substitutions in the RNA molecule should show reciprocal effects to the ones described above. Fig. 6 shows a series of G20-derived RNA molecules with single deoxyribonucleotide replacements around the cleavage site.
site. As before, the major cleavage site at nucleotide 11 was maintained independently of the deoxyribonucleotide position. There was no shift in the cleavage site position, even when a single deoxyribosyl moiety was introduced at the preferred cleavage site, nucleotide 11 (Fig. 6, G20–11D). This chimeric molecule was cleaved at the deoxyribonucleotide position 11 with strong selectivity over any ribonucleotide upstream or downstream. Whereas a deoxyribonucleotide at nucleotide position 12 only had a very small, if insignificant effect on the cleavage rate, there was a 2–3-fold downward shift in activity after replacement of nucleotide 11 with a deoxyribonucleotide (G20–11D). The nucleic acid with deoxyribonucleotides at both positions 11 and 12, G20–11,12D, was cleaved much less efficiently and showed a 11-mer formation rate very close to that of dG20. In addition to the low efficiency of cleavage at position 11 of G20–11,12D, this endonuclease substrate showed an increased number of alternative cleavage sites along the sequence. The increase in unspecific RNA hydrolysis became more apparent with more highly labeled G20–11,12D preparations (compare Figs. 5 and 6). The background cleavage results in relatively low final concentrations of 11-mer product with G20–11,12D as compared with other substrates. The G20-derived RNA 7A16A was again included as an independent standard of RNA cleavage rates. These results demonstrated that although the binding register of the G20-derived sequences was not influenced by either the presence or absence of 2'-hydroxy groups, the single nucleotides on each side of the cleavage site were major determinants of the apparent cleavage rate.

**Discussion**

The Level of RNA/DNA Discrimination in the Influenza Virus Endonuclease Active Site Is Low—This work addressed the problem of DNA/RNA discrimination by single strand-specific nucleases. The endonuclease of influenza virus constitutes an activity that is closely linked to the polymerase activity of the virus-encoded RNA-dependent RNA polymerase as well as to a specific cap-binding site on the polymerase complex. In the context of the complete trimeric influenza virus polymerase complex, the endonuclease cleaves RNA molecules to generate primers for transcription initiation. This activity appears to be tightly controlled by allosteric effects, since it is dependent on binding of a cap structure to the cap binding site on the PB2 subunit. But a high affinity binding site for the cap structure on the polymerase complex is only exposed after binding of a conserved promoter RNA sequence to the subunit PB1 (5, 31–33). Previous studies of the influenza virus endonuclease suggested a two-metal ion mechanism for this active site, analogous to the active site of the Klenow fragment exonuclease (17, 27, 34). However, in the crystal structure of the complex between single-stranded DNA and the Klenow exonuclease domain, the catalytic metal ions are not in proximity to the ribose 2'-position (35–37), and the Klenow fragment exonuclease readily hydrolyzes both RNA and DNA substrates (e.g. Fig. 1, lane K). To further elaborate the comparison between the influenza virus endonuclease and the Klenow model active site, we were therefore interested in determining how the influenza virus polymerase complex achieved the apparent specificity for RNA cleavage, which is observed in vivo.

Surprisingly, with the G20 sequences used in this study, the single-stranded DNA molecules were efficiently cleaved at the same position (dG11) as the corresponding RNA molecules (G11). This suggested that the endonuclease active site itself only had a limited ability to distinguish RNA from DNA molecules. The cleavage rate of dG20 DNA was about 5-fold lower than the rate of G20 RNA. However, RNA-like cleavage rates could be obtained in DNA molecules containing single ribonucleotides on both sides of the cleavage site. This magnitude of RNA/DNA discrimination makes it unlikely that the 2'-hydroxy group is involved in interactions directly contributing to catalysis of the chemical reaction. Mutagenesis experiments with other metal-dependent nucleases have shown at least 2–3-magnitude larger effects on catalysis upon removal of monodentate metal ion ligands in the active site (36, 38–50). One nucleotide, where evidence for a direct interaction of a metal ion with the 2'-hydroxy group has been obtained, RNase P, shows a more than 3000-fold reduction of cleavage rate when a deoxyribonucleotide was introduced at the cleavage site (24). Therefore, we consider it unlikely that the 2'-hydroxy group is a ligand of one of the catalytic metal ions in the endonuclease active site.

The present results are more consistent with a model in which the 2'-hydroxy group at the cleavage site has only an indirect conformational influence on the catalytic step. For example, it could reduce the energy barrier for the nucleic acid substrate to adopt a ribose conformation geometrically aligned for the chemical reaction by favoring a C3'-endo conformation of the ribose at the cleavage site.

Cleavage Site Choice at Position 11 of (d)G20 Substrate Is Dominant over Ribonucleotide Preference at the Cleavage Site—We were also surprised to find that by replacing single deoxyribonucleotides along the dG20 DNA molecule with ribonucleotides, we could not induce cleavage activity at the single ribonucleotide position. It has been reported with other RNA substrates that the influenza virus endonuclease, although functionally linked to the cap binding site, could cleave RNA molecules at variable distances 9–15 nucleotides from the cap (7, 9–12, 30, 51–53). Hagen et al. (30) showed that the endonuclease could alter cleavage site choice when single bases were changed on a model RNA substrate similar to G20 RNA. In the present study, the major cleavage site at (d)G11 was always retained on all DNA and RNA substrates we examined containing the G20 base sequence. Only in some cases were alternative cleavage sites observed. The major alternative cleavage site was at the guanosine residue G16. But the extent to which cleavage occurred at this site was independent of the status of the 2'-position on the ribose of G16.

The cleavage rates of the DNA molecules containing single ribonucleotide exchanges were virtually identical to the rates of complete DNA molecules except in the cases where positions 11 and 12 were affected. None of the chimeric molecules we studied showed cleavage at (d)G11 with a rate higher than G20 RNA or lower than the 5-fold reduction observed with dG20.
DNA. These results suggested that the binding of DNA, RNA, and chimeric molecules in the area between cap structure and cleavage site was independent of any conformational effects or interactions mediated by the 2'-hydroxy groups. In all cases, the nucleic acid substrate was kept in an identical position relative to cap binding and endonuclease active sites. This suggests that the cleavage at deoxyribonucleotide dG11 was significantly faster than a rearrangement of the substrate nucleic acid on the endonuclease protein to insert ribonucleotides upstream or downstream of position 11 into the active site.

These results appear to be in contrast to the observations of Olsen et al. with a different RNA substrate sequence. They found that they could virtually abolish cleavage activity at the preferred position 13 on an avian myeloblastosis virus-derived RNA oligonucleotide by replacing the ribonucleotide at position 13 with a deoxyribonucleotide. With this molecule, the cleavage site was instead shifted to position 14, and the cleavage efficiency appeared to be reduced (27). Why the endonuclease behaves differently on the avian myeloblastosis virus RNA-derived oligonucleotide is not clear at the moment, but it could be related to the very unusual U-rich sequence of the avian myeloblastosis virus RNA with U represented by 14 out of 18 nucleotides. It is possible that U-rich sequences bind differently to influenza virus polymerase as compared with mixed sequence oligonucleotides. The fact that poly(U) is a surprisingly strong inhibitor of endonuclease activity when compared with other homopolymers and unspecific RNAs is consistent with this possibility (54).

The Cap Binding Site, but Not the Endonuclease Active Site Is the Major Determinant of Single-stranded Nucleic Acid Discrimination—Within the fixed binding register of (d)G20 RNA and DNA substrates on the polymerase complex, the ribonucleotides at positions 11 and 12 measurably regulated the rate of nucleic acid cleavage within a 5-fold window of activity. Either RNA molecules with single deoxyribonucleotide substitutions or DNA molecules with single ribonucleotide substitutions gave corresponding results, demonstrating that position 11 had the major effect on nuclease activity with a more than additive contribution from position 12. This could be explained if the ribose conformation at position 11 had the principal influence on the geometry of the target phosphate between bases 11 and 12 with a smaller contribution of position 12 2'-OH toward the same preferred phosphate conformation.

Nevertheless, if the endonuclease active site were the only determinant of nucleic acid selectivity, a significant amount of DNA would still be hydrolyzed by this enzyme in a mixture of RNA and DNA molecules. But the influenza virus endonuclease is also regulated by the cap binding site of the protein. Neither RNA nor DNA cleavage could be detected under the present in vitro conditions if these nucleic acids lacked a cap structure or if they lacked m7G at the 5'-end. We estimated that the cleavage rate of uncapped RNA must be at least 2 orders of magnitude slower than cleavage of capped DNA (data not shown). The dependence of the influenza virus endonuclease on methylated cap structures has been reported before for a different substrate (10).

These results suggest that the influenza virus endonuclease has not been optimized to distinguish RNA from DNA, but rather to recognize capped nucleic acids. With no capped DNA molecules present in the cellular environment, this specificity ensures the use of RNA substrates for cleavage and the use of capped RNA primers for transcription initiation. It may therefore be speculated that the influenza virus RNA-dependent RNA polymerase has recruited and combined a prototypical low specificity two-metal ion nuclease domain and a cap binding domain to create the unique system of cap snatching for the highly specific generation of capped viral mRNAs.

Transcription Initiation from DNA Primers—The specificity of DNA cleavage by the influenza virus endonuclease was supported by the efficient use of the DNA primers for transcription initiation in the presence of CTP. Since CTP-dependent initiation has so far only been observed with primers containing guanosine residues at the 3'-end, these observations are consistent with the mapping of the major cleavage site to guanosine residue (d)G11 with all nucleic acid substrates of
this study irrespective of ribo- or deoxyribonucleotide positions elsewhere on the molecule.

All substrates supported transcription initiation with CTP. In a preliminary analysis of this reaction under single turnover conditions, we observed a significant increase in the apparent $K_m$ value for CTP with dG11 DNA primers, whereas the apparent initiation rates ($k_{app}$) were very similar with DNA and RNA primers (Table I). This is consistent with a model where the presence of a deoxyribonucleotide in the polymerase active site interfaces with the binding of the next NTP substrate rather than with the catalytic step. It may be possible that a ribonucleotide at the 3'-end of the primer takes up a conformation that allows more efficient base stacking with the incoming NTP. More experiments are required to test this model and to investigate the influence of further nucleotide modifications on binding interactions in the polymerase active site. Interestingly, when we added only a single 2'-hydroxyl group to the 3'-terminal nucleotide we obtained a primer molecule with an apparent $K_m$ value for CTP even lower than the $K_m$ value in the presence of G11 RNA and with no apparent change in the initiation rate.

In the combined endonuclease/transcription initiation reactions in the presence of dG20 nucleic acids and CTP, the apparent rate of transcription initiation was about 5-fold lower with dG20 DNA compared with G20 RNA, similar to what had been observed in the endonuclease reaction. This indicated that under these conditions the cleavage reaction was rate-limiting. The apparent $K_m$ value for CTP was again higher with the DNA substrate and could be lowered to a value close to the one obtained with G20 RNA by adding a 2'-hydroxyl group to nucleotide 11 of the dG20 DNA.

Together these results showed that capped, single-stranded DNA molecules were efficient substrates for both endonuclease and polymerase active sites during transcription initiation in vitro. The main determinants for RNA selectivity were the cap structure and the binding of two nucleotides in the endonuclease active site. The binding of the spacer sequence between these two sites was independent of 2'-hydroxyl group to RNA primers (Table I). This is consistent with a model where a ribonucleotide at the 3'-end of the primer takes up a conformation that allows more efficient base stacking with the incoming NTP. More experiments are required to test this model and to investigate the influence of further nucleotide modifications on binding interactions in the polymerase active site. Interestingly, when we added only a single 2'-hydroxyl group to the 3'-terminal nucleotide we obtained a primer molecule with an apparent $K_m$ value for CTP even lower than the $K_m$ value in the presence of G11 RNA and with no apparent change in the initiation rate.

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