Comparative Properties of RNA and DNA Templates for the DNA Polymerase of Rous Sarcoma Virus

(oligonucleotides/cellular RNAs/viral RNAs/purified enzyme)

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ABSTRACT Several natural RNAs were compared with respect to their template activities for the DNA polymerase of Rous Sarcoma Virus during a 2-hr incubation period. 60-70S viral RNA was found to be a 5- to 10-fold better template than heat-dissociated Rous viral RNA, influenza virus RNA, tobacco mosaic virus RNA, or ribosomal RNA. Denatured salmon DNA is a little better, and poly(dAT) is 2-4 times better as a template for the enzyme than is 60-70S Rous viral RNA. The 60-70S RNAs of different strains of avian tumor viruses have very similar template activities for a given avian tumor virus DNA polymerase. Oligo(dT) or oligo(dC) were found to enhance the template activity of heat-dissociated Rous viral RNA 20- to 30-fold, and that of other natural RNAs tested one- to several-fold. DNA syntheses of 1-24% were obtained during a 2-hour incubation of the enzyme with the above RNA templates. The results suggest that the enzyme prefers partially double-stranded or hybrid regions of RNAs for optimal DNA synthesis, but certain regions of single-stranded RNA can also serve as templates.

Poly(dAT) competes with viral RNA for purified DNA polymerase during DNA synthesis, as would be expected if RNA- and DNA-dependent DNA synthesis was performed by at least one common active site of the same enzyme.

Indirect evidence suggests that RNA tumor-virus replication requires virus-specific DNA early in infection (1, 2). This virus-specific DNA is thought to be transcribed from viral RNA by the virus-associated DNA polymerase (3, 4). The ubiquity of this enzyme in all known RNA tumor-viruses (3-5), its ability to transcribe *in vitro* most or all viral RNA (6, 7), and the presence of at least one enzyme per virion (8) are compatible with its role of a RNA-DNA transcriptase essential for virus replication. Very different kinds of nucleic acids, however, were reported to be templates for virus-associated DNA polymerase, perhaps indicating that Rous Sarcoma Virus (RSV) DNA polymerase is not specific for virus replication. Unpurified DNA polymerase of RNA tumor-viruses was shown to accept exogenous natural DNA (9-11), synthetic DNA, DNA-RNA hybrids, or double-stranded RNA as templates (10, 12) besides the endogenous viral RNA (4, 3, 6). Synthetic homopolymer RNAs, however, were found to be poor templates (12, 10), except when present with a complementary oligodeoxynucleotide primer (13). A purified DNA polymerase of RSV that was free of endogenous template responded to both natural DNA and single-stranded RNA templates, and preferred among natural RNAs the 60-70S RNA of RSV (8).

Abbreviations: RSV, Rous sarcoma virus; TMV, tobacco mosaic virus.

The present report describes quantitative comparisons of the template activities of various nucleic acids in the presence or absence of oligodeoxynucleotides for the purified RSV DNA polymerase. Further, the question of whether RNA and DNA templates compete for the same RSV DNA polymerase was investigated.

MATERIALS AND METHODS

[³H]dCTP, [³H]dTTP, 14-20 Ci/mmol; [³H]dATP, 6 Ci/mmol were purchased from New England Nuclear, [³H]dGTP 3 Ci/mmol from Amersham, deoxynucleotide triphosphates from Sigma or CalBiochem, and oligodeoxyribonucleotides (of chain length 12-18) from Collaborative Research Inc., Waltham, Mass. Poly(dAT) was a gift of Dr. M. Chamberlin.

Viruses. Prague RSV of subgroup (14) C was used to prepare (8) 60-70S RNA and DNA polymerase, unless otherwise indicated. DNase treatment of RSV RNA (8) was in 5 mM MgCl₂-5 mM KCl-50 mM Tris (pH 7.4). Schmidt-Ruppin RSV of subgroup A, myeloblastosis-associated virus of subgroup B, and avian leukosis MC-29 virus of subgroup A were all originally obtained from Dr. P. K. Vogt (14) and grown as described (8).

Standard DNA Polymerase Assay. Purification of the soluble RSV DNA polymerase (8) was in a 20-40% (v/v) glycerol gradient. The enzyme was stored at -20°C in the gradient solution after the addition of glycerol to a final concentration of 50%.

Standard assays were at 40°C for 2 hr in 100 μl of solution containing 50 mM KCl, 50 mM Tris (pH 8.0), 5 mM dithiothreitol, 6 mM MgCl₂, three unlabeled dNTPs at 0.1 mM, and one [³H]dNTP at 1.25 μM (8). The assay contained 15 μl of enzyme solution. Given a 20-fold purification of the enzyme, based on the total soluble protein of the virus (8) and the assumption that 2 A₂₆₀ (measured in 0.2% sodium dodecyl-sulfate) of purified virus corresponds to 1 mg of protein (8), the protein content of 15 μl of enzyme solution was 50-75 ng, which was operationally defined as one protein unit.

RESULTS

Dependence of DNA synthesis on enzyme and template concentration

Constant concentrations of 60-70S RSV RNA, heat-dissociated RSV RNA (15), influenza RNA, or tobacco mosaic virus RNA were incubated with different concentrations of RSV DNA polymerase. Maximal [³H]DNA synthesis

FIG. 1. Dependence of RNA-primed $[^3\text{H}]$ DNA synthesis by RSV DNA polymerase on enzyme concentration. 1 μg of the indicated RNA was incubated under standard assay conditions, but with different amounts of protein units (*Methods*) of purified enzyme.

was obtained in all cases with 2 protein units of enzyme (Fig. 1), 5 times more DNA was synthesized at saturating concentrations of enzyme with 60-70S RSV RNA than when heat-dissociated RSV RNA or other RNAs were used as templates. This result is compatible with the hypothesis that a defined structure of the RNA template determines the rate of $[^3\text{H}]$ dCTP incorporation by RSV DNA polymerase.

Saturation quantities of a constant concentration (half-

saturation for 1 μg of RNA, see Fig. 1) of RSV DNA polymerase with several nucleic acids are shown in Fig. 2. It appears that all nucleic acids reach, or at least approach, plateaus of maximal DNA synthesis at concentrations of 1-5 μg . Particularly with TMV RNA, but also with 28S ribosomal RNA and influenza RNA (Fig. 2B), the saturation curves suggest that there is inhibition of the enzyme by high concentrations of RNA templates. Heating of ribosomal RNA to 100°C, followed by sedimentation, did not affect its template activity, (not shown). Among the RNAs, 60-70S RSV RNA stimulates incorporation of $[^3\text{H}]$ dCTP 5- to 10-times better than all single-stranded RNAs tested, including heat-dissociated RSV RNA. The template activity of denatured salmon DNA (8) was about 1.3 times, and that of poly (dAT) about 3 times (see below), higher than that of 60-70S RSV RNA.

Kinetics of DNA synthesis with several RNA templates

The kinetics of DNA synthesis with several RNA templates are shown in Fig. 3. All RNAs with high template activities, like 60-70S RSV RNA or RNAs in the presence of oligodeoxynucleotides (see below), exhibit relatively high rates of DNA synthesis early, and decreasing rates of DNA synthesis late, in an assay period of 2 hr. Heated RSV RNA stimulates DNA synthesis at a lower rate than does 60-70S RSV RNA, but the kinetics are similar to those obtained with 60-70S RSV RNA.

The kinetics of DNA synthesis with TMV RNA and 28S ribosomal RNA, however, show an initial lag period, but are otherwise similar to those obtained with the other RNAs. Despite these differences, the kinetics of DNA synthesis with various RNA templates are considered to be similar enough to justify a comparison of their template activities for RSV polymerase on the basis of the DNA yield that is obtained under standard assay conditions.

FIG. 2. Dependence of $[^3\text{H}]$ DNA synthesis by RSV DNA polymerase on template concentration under standard assay conditions. If indicated, 1 μg of oligo (dT) or (dC) was used per assay. Poly(dAT)-dependent $[^3\text{H}]$ DNA synthesis was determined at a $[^3\text{H}]$ dTTP concentration of 20 μM . Ribosomal RNA was prepared from a cytoplasmic extract (21) and twice treated with DNase, followed by sucrose gradient sedimentation.

Comparisons of the template activities of the RNAs of different avian tumor viruses

To determine whether the template activity of 60-70S RNA (Prague strain) for its homologous DNA polymerase was strain- or subgroup-specific (14), or whether all avian tumor virus RNAs have the same template activity for a given avian tumor virus DNA polymerase, the comparisons summarized in Table 1 were made. It can be seen that within the limits of accuracy of such assays, all 60-70S avian tumor virus RNAs have very similar template activities for a given enzyme.

Effects of oligodeoxynucleotides on template activities for RSV DNA polymerase

As shown in Figs. 2 and 3 and Table 2 the template activities of natural RNAs were increased by oligo(dT) or oligo(dC) 1- to several-fold. The template activity of heat-dissociated RSV RNA was increased 20- to 30-fold by oligo(dT) or by oligo(dC), and about 10-fold by oligo(dG). Oligo(dA) did not affect the template activity of heated RSV RNA. It appears that oligo(dT) or oligo(dC) restore the high template activity of RSV RNA that is lost after heat-dissociation to about the template activity of native 60-70S RSV RNA in the presence of oligo(dT) (Fig. 2). The template activity of TMV RNA was slightly stimulated by oligo(dT), but was stimulated about 10-fold by oligo(dC) (Fig. 3). Preliminary analyses indicate that the sizes of the DNAs produced in the presence or absence of oligodeoxynucleotides are similar.

About 1 μ g of oligo(dT) per standard assay was necessary to maximize DNA synthesis directed by 1 μ g of RNA template (experiments not shown). Oligo(dT) or oligo(dC) with-

TABLE 1. Comparison of the template activities of different avian tumor virus RNAs for the purified DNA polymerases of different strains of avian tumor viruses

DNA polymerase of	RNA template	[³ H]dTMP incorporated cpm
SR RSV-A*	SR RSV-A*	4804
	PR RSV-C†	3312
PR RSV-C†	PR RSV-C	62798
	SR RSV-A	48170
	MC29-A§	50852
	TMV	4826
MC29-A§	MC29-A	11402
	PR RSV-C	13862
	SR RSV-A	14374
	TMV	573
MAV-B‡	MAV-B‡	6322
	PR RSV-C	10271
	MC29-A	10782
	SR RSV-A	5005
	TMV	455

0.5 μ g of template 60-70S tumor virus RNA or TMV RNA was assayed under standard conditions. Various amounts of enzymes were used, and are not equivalent for different viruses.

* Schmidt-Ruppin strain, subgroup A.

† Prague strain, subgroup C.

‡ Myeloblastosis-associated virus, subgroup B.

§ Avian leukosis virus, subgroup A.

FIG. 3. Kinetics of [³H]dCTP incorporation by RSV DNA polymerase with 1 μ g of the indicated RNA templates. 1 μ g of oligodeoxynucleotide was added when indicated.

TABLE 2. Effects of oligodeoxynucleotides on the template activities of RSV RNA or KOH-denatured salmon DNA for RSV DNA polymerase under various conditions

Standard assay* with indicated [³ H]dNTP	Template (1 μ g)	Oligo (dN) (1 μ g)	[³ H]dNMP incorporated, cpm (average $\pm$ range)§
[³ H]dCTP	60-70S RSV RNA	...	103,850 $\pm$ 7000
	heated RSV RNA	...	7,200 $\pm$ 500
	same	(dT)	185,060 $\pm$ 12000
	same	(dA)	7,800 $\pm$ 800
	same	(dC)	257,081 $\pm$ 30000
	same	(dG)	72,508 $\pm$ 2050
[³ H]dTTP	60-70S RSV RNA	(dT)	333,990 $\pm$ 16250
	same	...	107,510
	same	(dT)	1,030
	same, -dATP	(dT)	110,216 $\pm$ 4900
[³ H]dTTP†	60-70S RSV RNA	...	500
	poly(dAT)‡	...	108,405
	same, -dATP	...	500
	same, -dTTP	...	500
[³ H]dCTP	60-70S RSV RNA	(dT)	42,056 $\pm$ 5000
	same, -dATP	(dT)	306
	same, -dTTP	(dT)	270
	same, -dGTP	(dT)	7,532
[³ H]dGTP	same	(dT)	219
	same, -dTTP	(dT)	32,375 $\pm$ 450
	same, -dATP	(dT)	1,012
	same, -dGTP	(dT)	822
[³ H]dTTP	salmon DNA	...	76,226 $\pm$ 800
	same	(dT)	79,480
	same, -dATP	...	7,733

* The activities per unit of protein of different polymerase preparations varied about 2-3 fold in different experiments.

† [³H]dTTP was 2.6 μ M, dGTP and dCTP were omitted.

‡ 0.3 μ g. § of two determinations.

Fig. 4. Competition of 60-70S RSV RNA and poly(dAT) for RSV DNA polymerase. A. RSV RNA-dependent [³H]DNA synthesis was determined under standard assay conditions in the presence of 2 µg of 60-70S RNA, with or without oligo(dT), and different concentrations of poly(dAT). B. After incubation of RSV DNA polymerase for 30 min with 3 µg of poly(dAT) in a standard assay solution at 40°C, incubation was continued for 2 hr after the addition of various concentrations of 60-70S RNA, with and without 1 µg of oligo(dT).

out RNA (Table 2) or added after RNase treatment of a RNA template (not shown) did not function as a template for DNA synthesis. Oligo(dT) did not affect the template activity of KOH-denatured salmon DNA (8) (Table 2) or poly(dAT) (not shown).

Results of substrate deletion experiments are summarized in Table 2. RSV RNA-dependent DNA synthesis, measured by incorporation of [³H]dTTP in the presence of oligo(dT), was inhibited 70% if dATP was omitted. RSV RNA-dependent DNA synthesis in the absence of oligo(dT), as well as poly(dAT)-dependent DNA synthesis, were inhibited 99.5% if dATP was omitted, thus indicating that the [³H]-dTTP was not contaminated by dATP. If RSV RNA-dependent DNA synthesis in the presence of oligo(dT) was measured by incorporation of any of the other three [³H]dNTPs, however, there was 97% inhibition if an unlabeled dNTP was omitted. These results are compatible with the synthesis of a T-rich polydeoxynucleotide as well as virus-specific DNA (7) in the presence of 60-70S RSV RNA and oligo(dT), and imply that there may be an A-rich sequence of RSV RNA.

Amount of [³H]DNA synthesized in the presence of various templates

Under standard assay conditions, the amount of [³H]DNA formed (at a counting efficiency of 25% for ³H) by RSV DNA polymerase, relative to the 60-70S RSV RNA template, was 4-6%, and as much as 12% in the presence of oligo(dT) or oligo(dC) (Figs. 1 and 2, Table 2); it doubled if the molarity of [³H]dCTP was raised from 1.25 µM to 50 µM (not shown). With TMV RNA, ribosomal RNA, or influenza virus RNA, [³H]DNA synthesis was ≤1% relative to the template added; more DNA was made in the presence of oligo(dT) or oligo(dC) (Fig. 2). DNA synthesis with a poly(dAT) template was about 50% or more at a [³H]dTTP concentra-

tion of 26 µM (Table 2). Since the above estimates were obtained at high or saturating concentrations of enzyme, we can conclude that the amount of DNA formed relative to the RNA templates added is low under our conditions.

Competition of RNA and DNA templates for the DNA polymerase

To test whether RNA and DNA templates compete for the same RSV DNA polymerase, the following experiments were done. [³H]DNA synthesis was measured in the presence of constant, saturating (Fig. 2) amounts of 60-70S RSV RNA, with and without oligo(dT), and with different concentrations of poly(dAT). Only RSV RNA-dependent DNA synthesis is measured by incorporation of [³H]dCTP, although poly(dAT) is an excellent template for RSV DNA polymerase (Fig. 2) (16). As shown in Fig. 4A, increasing concentrations of poly(dAT) reduced incorporation of [³H]dCTP into [³H]DNA. Further, if different concentrations of 60-70S RSV RNA were added to a standard assay solution during 30 minutes of poly(dAT)-dependent DNA synthesis, incorporation of [³H]dCTP into [³H]DNA increased (Fig. 4B). These results indicate that 60-70S RSV RNA, particularly in the presence of oligo(dT), can displace poly(dAT) from RSV DNA polymerase, and that both templates have similar affinities for the enzyme.

DISCUSSION

The finding that poly(dAT) inhibits RSV RNA-dependent DNA synthesis by RSV DNA polymerase is consistent with the hypothesis that RNA- and DNA-dependent DNA syntheses are performed by at least one common active site of the same enzyme or enzyme complex. This result is compatible with previous observation that RNA- and DNA-dependent polymerase activities of RSV coincide in sucrose gradients (8) and phosphocellulose columns (v. d. Helm, unpublished). It is possible, however, that RNA- and DNA-dependent DNA syntheses occur at different active sites, and that DNA acts as a template at one site and blocks RNA-dependent DNA synthesis at the other.

It seems likely that the increase of template activities of RNAs with oligo(dT) or oligo(dC) is due to their interaction with the RNA template. This can be deduced from the observations that oligo(dT) or oligo(dC) are, by themselves, without significant template activity and have different effects on the template activities of different RNAs. Further work will be necessary to decide whether oligo(dT) and oligo(dC) interact with complementary regions of the RNA template, as suggested by analogy to the experiments of Baltimore and Smoler (13), or whether there is also some interaction with the enzyme.

As a tentative explanation for the results that at (probably) saturating concentrations of the enzyme (Fig. 1), or of RNA template (Fig. 2), 60-70S RSV RNA has a 5- to 10-fold higher template activity for RSV DNA polymerase than do heat-dissociated RSV RNA and the other RNAs tested, the following hypothesis may be suggested. Two different types of RNA structure determine the template activity of a given RNA: partially double-stranded regions, which probably exist in 60-70S RSV RNA (15) or hybrid regions, which might exist in the presence of oligodeoxynucleotides, are required for optimal binding to the enzyme and serve as optimal starting points; they thus account for the high rate of DNA synthesis. Certain regions in single-stranded

RNAs, such as a self-complementary loop, may resemble partially double-stranded RNA and stimulate DNA synthesis at a lower rate. The low yields of DNA synthesis obtained even with high concentrations of single-stranded RNAs may be due to enzyme inhibition. At high concentrations of single-stranded RNA, the enzyme may not be permitted to transcribe a maximal region of the RNA template, because it may be displaced (e.g., Fig. 4) by binding sites of an RNA that is unable to initiate DNA synthesis. Partially double-stranded regions may also account for the high template activities of denatured salmon-DNA or poly(dAT).

It is conceivable that with partially double-stranded RNA, one strand serves as a template and the other as a primer, which may be incorporated in the DNA product. Preliminary experiments on characterization of DNA product obtained with 60-70S viral RNA as template after brief synthesis (unpublished) suggest that at least some DNA product is covalently linked to RNA after velocity or isopycnic sedimentation in 1.1 M CH_2O . These results are compatible with those of D. Baltimore and J. Hurwitz (personal communication).

An interpretation of the result that low amounts of RNA are formed by RSV DNA polymerase relative to the RNA templates tested cannot as yet be given. It may be due in part to an obligatory primer requirement.

The template requirements of RSV DNA polymerase suggest that the enzyme resembles bacterial DNA polymerase, which is known to prefer a partially double-stranded DNA template (17) and to transcribe single-stranded DNA optimally in the presence of an oligodeoxynucleotide primer (18). However, bacterial DNA polymerase, which has been shown to accept RNA templates (19, 13, and 22), and RSV DNA polymerase may differ with respect to template specificities for RNA.

The essential question as to whether RSV-associated DNA polymerase is involved in virus replication (4, 3) cannot be answered definitively on the basis of its known *in vitro* properties. Some properties that favor its role as a replicase are listed in the introduction. Other *in vitro* properties of the enzyme, including the relatively low synthesis of DNA with the RNA templates tested and the rather small sizes of the *in vitro* DNA products (20, 6, 10), do not support its putative role of a virus-specific RNA-DNA replicase, but these properties may be due to experimental artefacts of the *in vitro* systems.

NOTE ADDED IN PROOF

An adenylate-rich region has been found in RSV 70S RNA (M. Lai, unpublished data).

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