Effect of Chemically Modified 70S RNA from Avian Myeloblastosis Virus (AMV) upon the Activity of AMV DNA Polymerase

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Adenine residues of 70S avian myeloblastosis virus (AMV) RNA are modified when reacted with chloroacetaldehyde. This modification introduces characteristic fluorescent ϵ -adenosine (ϵ A) probes which were used to monitor the reaction. Under suitable conditions, modified 70S(ϵ A) RNA was maintained intact and was inactive as a template for the AMV DNA polymerase. Furthermore, it inhibited the reaction catalyzed by AMV polymerase when 70S RNA was used as template-primer and had no effect on the two tested bacterial polymerases. Protection against the 70S (ϵ A) RNA inhibition was observed when 70S RNA was primed with oligo(dT) indicating preference of the polymerase for the oligo(dT) primed regions.

There have been several reports on the use of polyribonucleotides and their analogues as inhibitors of oncornavirus DNA polymerases. Polymer studies included polyadenylic acid, poly(2'-*o*-methyl adenylic acid) (16) and polyuridylic acid (5, 16, 17). Interest in these inhibitors has been amplified by reports that certain polymers inhibit viral replication (15, 16). Synthetic polymers have also been exploited as probes to study the mechanism of catalysis by DNA polymerases. In view of some evidence that viral DNA polymerases are agents in the onset of viral expression, its selective inhibition has the potential of providing an extremely important tool in suppressing oncogenic information (7; M. A. Chirigos and T. Papas, Academic Press Inc., in press). In general, no single-stranded polymers can serve as substrates for the polymerase reaction unless primed with their respective complementary nucleotides (4, 9) possessing a 3'-terminal hydroxyl group. Despite several studies dealing with synthetic homopolymers, there have been no attempts to modify naturally occurring 70S RNA in an effort to render it an effective inhibitor. We initially reported modification of poly(A) and the effects of such modifications on poly(dT) synthesis catalyzed by avian myeloblastosis virus (AMV) DNA polymerase (J. G. Chirikjian and T. S. Papas, *Biochem. Biophys. Res. Commun.*, in press). We have now extended these studies to naturally occurring 70S RNA.

In this paper, we report the first chemical

modification of AMV 70S RNA under conditions that minimize degradation and have no effect on its sedimentation pattern. Such a modification was achieved by converting adenine residues to their fluorescent ϵ A analogue. The resulting 70S(ϵ A) RNA is highly fluorescent and inactive as a template for AMV DNA polymerase. In addition to being a useful analogue inhibitor of naturally occurring 70S RNA, it promises to be an important probe for studying interactions with the cognate DNA polymerase.

MATERIALS AND METHODS

Reagents. Unlabeled nucleoside triphosphates and deoxytriphosphates were purchased from P-L Biochemicals. Tritiated nucleoside triphosphates and deoxytriphosphates were obtained from Schwarz-Mann; specific activities (curies per millimole) were: dTTP, 17.3; dATP, 20.9; dCTP, 30; dGTP, 20; UTP, 12. Whatman phosphocellulose P-11 was from H. Reeve Angel, Inc. Calf thymus DNA and DNase I were from Worthington Biochemicals, Corp. Nonidet P-40 (NP-40) was obtained from Shell Chemical Co. (dT)₁₂₋₁₈ was purchased from Collaborative Research.

Virus. AMV was obtained from the plasma of infected chicks provided through contract NO1CP33291 within the Virus Cancer Program of the National Cancer Institute. Cell debris was removed by centrifugation at $600 \times g$, followed by a 30-min centrifugation at $5000 \times g$. The virus was further concentrated and purified as described (12).

Preparation of 70S AMV RNA. High molecular weight 70S RNA from AMV was extracted (13), and layered onto linear sucrose gradients of 5 to 30%

(wt/wt) in 0.1 M NaCl, 0.01 M Tris (pH 7.3), and 0.001 M EDTA. Centrifugation was performed for 3 h at $105,000 \times g$. Fractions were collected and precipitated with ethanol. External markers used were 28 and 18S [3 H]RNA from NC-37 cells.

[14]uridine-labeled AMV virus was kindly supplied by Danny P. Bolognesi of Duke University. [14]uridine 70S RNA was extracted as previously described above.

Chemical modification of 70S RNA with chloroacetaldehyde. Chloroacetaldehyde was prepared by hydrolysis of its dimethylacetal (Aldrich) in dilute sulfuric acid, and the liberated aldehyde was collected by distillation (14). The pH of the distillate was adjusted to 7.4 with KOH and was stored at -20°C .

Modification of adenine residues of 70S RNA was carried out by incubating the RNA in the presence of chloroacetaldehyde, pH 4.5, (0.01 to 0.1 M) at 37°C for 1 h. Samples were lyophilized to remove the reagent.

Fluorescent measurements. A Hitachi Perkin Elmer MPF-2A Spectrofluorometer equipped with a thermostated cell holder was used for all measurements with a 5-nm band width for both excitation and emission. All measurements were performed using dilute solutions to avoid correction for inner filter effects. In addition, correction for scattering was made when necessary.

Enzymes. AMV DNA polymerase was assayed essentially as described (15). The reaction mixture contained in a final volume of 0.1 ml: 50 mM Tris-hydrochloride (pH 8.3), 6 mM MgCl_2 , 0.4 mM dithiothreitol, 50 mM KCl, 0.2 mM each of dATP, dGTP, and dCTP, 0.125 mM [^3H]TTP (80 to 300 counts per min per pmol), (dT) $_{12-18}$ and 70S RNA as designated in the appropriate tables and figures. The oligo(dT):70S RNA ratio was 1:5 by weight (8). Reactions were incubated at 37°C for 30 min and stopped by transfer to 4°C . To each reaction 0.2 μg of bovine serum albumin, 0.2 μg of yeast RNA, and 10 μmol of pyrophosphate were added followed by cold trichloroacetic acid to final concentration of 10%. After allowing the reaction mixtures to incubate for 10 min at 4°C , acid-insoluble material was collected on Whatmann GF/C filters. Filters were washed with 10% trichloroacetic acid and the radioactivity was counted. Transcription of 70S RNA both in the absence and presence of oligo(dT) was dependent upon presence of all four deoxytriphosphates. Omission of dCTP or dGTP reduced DNA synthesis by 80%, indicating that the TMP incorporation involved heteropolymer DNA formation, and not transcription of only the poly(A) region of 70S RNA. The product of the reaction was alkali and RNase stable but sensitive to DNase.

The purified fraction of AMV DNA polymerase showed only two distinct bands in SDS disc gel electrophoresis, representing the two nonidentical subunits. Purified enzyme was used for all subsequent studies.

DNA-dependent RNA polymerase from *Escherichia coli*, a gift from Robert Lazzarini, was assayed and purified by established procedures (2).

DNA-dependent DNA polymerase from *Micrococ-*

cus luteus was purchased from P-L Biochemicals and assayed by published procedures (3).

RESULTS

Sedimentation pattern of AMV 70S RNA.

Total RNA extracted from AMV virus was sedimented in a sucrose gradient (Fig. 1). The gradient was monitored for absorbance at 260 nm and fractions were assayed for template-primer activity by using purified AMV DNA polymerase. The activity was found to be associated only with the major absorption peak which sedimented in the region of 70S. In agreement with previous reports, at least a six-fold stimulation of 70S RNA activity was noted when synthetic oligo(dT) was added to reaction. There was no activity associated with RNA fractions that sedimented in regions other than 70S RNA. In a typical experiment about 50% of the total RNA sedimented in the 70S region. For preparative work the 70S peak was precipitated with ethanol, dissolved in water, and stored frozen at -90°C . Under these storage conditions, 70S RNA was maintained intact for several months as checked by its sedimentation profile and its activity as a template primer for the polymerase. These two criteria were routinely checked prior to chemical modification of RNA samples.

Chemical modification of 70S RNA with chloroacetaldehyde. It has been reported that a solution of chloroacetaldehyde selectively modified adenine residues at pH 4.5, whereas at pH 3.5 only cytosine residues were modified (1). As shown in the diagram of the structures of the

FIG. 1. Sedimentation profile of 70S RNA extracted from AMV virus, as recorded by absorbance and DNA polymerase activity. Solid line represents the absorbance tracings at 260 nm. Symbols: O, activity in the absence of oligo(dT) primer; ●, activity upon addition of oligo(dT) primer.

modified nucleosides (Fig. 2), they are not capable of base pair formation and can be distinguished from each other by their characteristic emission maxima.

The fluorescence spectrum of 70S RNA modified by chloroacetaldehyde at pH 4.5 is shown in Fig. 3. An emission maximum at 408 nm was observed; this is very close to that of ϵ -AMP (1) and is in agreement with the maximum found for ϵ -poly(A) (11; J. G. Chirikjian and T. S. Papas, *Biochem. Biophys. Res. Commun.*, in press). In the absence of chloroacetaldehyde 70S RNA incubated at 37°C for 1 h showed no fluorescence between 350 and 550 nm. It should be noted that the fluorescence spectrum of 70S(ϵ A) RNA showed no contribution by ϵ -cytosine residues which show a maximum at 360 nm.

Sedimentation profile of [14 C]70S RNA after adenine modification. To evaluate the size of the modified 70S RNA, samples after 1.5 and 5.5 h of incubation with chloroacetaldehyde were analyzed on sucrose gradients. A single peak of RNA sedimenting in the 70S region was observed with the RNA that had been incubated for 1.5 h. However, after 5.5 h, degradation to fragments smaller than 35S were noted (Fig. 4). Samples of the 70S RNA that were modified by using 1-h incubation were used routinely in the ensuing studies.

Intactness of the phosphodiester bond in 70S (ϵ A) RNA was tested by heat denaturation.

Samples modified for one hour yielded RNA sedimenting at 35S (Fig. 5). The modification as monitored by fluorescence is associated mainly with 35S RNA implying that 70S RNA is the major target for ϵ A formation.

FIG. 3. Fluorescence spectrum of 70S (ϵ A) RNA after 1 h of modification. Measurements were carried out at pH 7.0 in aqueous solution where the RNA concentration was less than $1 A_{260}$ units. Corrections were made for scattering effects. Symbols: ●, 70S RNA treated with chloroacetaldehyde for 1 h at 37°C; ○, 70S RNA incubated for 1 h at 37°C in the absence of chloroacetaldehyde.

FIG. 2. Modification of adenine and cytosine residues when incubated with chloroacetaldehyde at pH 4.5 and 3.5, respectively.

FIG. 4. Sucrose gradient sedimentation of [^{14}C]uridine. Symbols: O, 70S unmodified RNA; Δ , 70S(ϵA) after 1.5 h of modification; $\square$, 70S(ϵA) after 5.5 h of modification; O, 70S RNA incubated at 37°C in the absence of chloroacetaldehyde. Direction of sedimentation is from left to right.

Effect of 70S (ϵA) RNA on DNA polymerase activity. The adenine modified 70S RNA was not used as a template by AMV DNA polymerase (Table 1). However, it completely inhibited the reaction directed by the unmodified 70S RNA. In contrast, the unmodified 70S RNA-dT₁₂₋₁₈-dependent reaction was only slightly inhibited by 70S (ϵA) RNA. Also shown in Table 1 is that 70S (ϵA) RNA did not effect the activities of either *E. coli* RNA polymerase or *M. luteus* DNA polymerase. In a separate experiment, a chloroacetaldehyde reaction mixture without 70S RNA was lyophilized, the residue dissolved in water, and then tested for inhibitory effect. No inhibition of AMV DNA polymerase was observed.

Effect of nucleic acids on 70S RNA directed DNA synthesis by AMV DNA polymerase. The inhibitory effect of nucleic acids containing chemically modified adenine residues were tested in the 70S RNA oligo(dT) primed or unprimed reaction (Table 2). Under the conditions tested, a greater degree of inhibition was observed by ϵ -poly(A) as compared with poly(A) (J. G. Chirikjian and T. S. Papas, Biochem. Biophys. Res. Commun., in press); also, 70S (ϵA) RNA was found to be a better inhibitor than ϵA -modified nonviral nucleic acids, which indicates a preference of the enzyme for its modified substrate analogue. When adenine-modified residues were introduced in tRNA (by

reaction with chloroacetaldehyde), no inhibitory activity was noted on the 70S RNA-directed transcription. This indicates that ϵA groups were only effective when introduced in critical regions required for the recognition process.

Relationship between the extent of modification and the inhibition of DNA polymerase. It was of interest to evaluate the degree of modification and relate this to the inhibitory activity. Modification, as monitored by fluorescence, was shown to plateau after 8 h and reached 50% of the plateau level after 1 h; no fluorescence was observed at zero time (Fig. 6). Portions, at indicated time intervals, were removed, and lyophilized to remove chloroacetaldehyde. Residues were dissolved in water and assayed in the polymerase reaction. A rapid loss of template activity was noted; after 1 h of modification, the 70S RNA lost 95% of its ability to act as a template both in the presence and absence of oligo(dT) (Fig. 6A). 70S RNA incubated in the absence of chloroacetaldehyde retained its full activity. Similarly, after 1 h of modification (Fig. 6B) almost total inhibition was observed of the reaction directed by unprimed 70S RNA. By contrast, when oligo(dT)-primed 70S RNA was used in the assay, 70% of the enzyme activity was resistant to inhibition by 70S(ϵA) RNA.

DISCUSSION

Evidence suggests that initiation of DNA synthesis on viral 70S RNA can occur at sites where naturally occurring 4S RNA primers are hydrogen bonded to 70S RNA (11), and at the poly(A) region of 70S upon priming with oligo(dT) (8). In each case, modification of selective residues in these regions can be of great

FIG. 5. Sucrose gradient sedimentation of [^{14}C]uridine modified and unmodified 70S AMV RNA after heating to 70°C for 3 min and centrifuged for 12 h at 105,000 $\times$ g. Symbols: Δ , 70S RNA incubated for 1 h in the absence of chloroacetaldehyde and then heated at 70°C for 3 min; $\bullet$, 70S RNA treated with chloroacetaldehyde for 1 h and then heated at 70°C for 3 min; $\bullet$, fluorescence intensity (arbitrary units).

TABLE 1. *Effect of AMV 70S(εA)RNA on the activity of DNA and RNA polymerases*

Reaction conditions	Amount of [³ H]dMP incorporated (pmol/30 min)	Activity (%)
A. DNA polymerase (AMV)		
(i) Template act ^a		
-Template	0.05	0
+70S(εA)RNA	0.37	6
+70S(εA)RNA·dT ₁₂₋₁₈	8.98	21
+70S RNA	6.2	100
+70S RNA·dT ₁₂₋₁₈	42.8	690
(ii) Inhibitory act		
+70S RNA + 70S RNA ^a	14.3	230
+70S RNA + 70S RNA ^b	14.9	241
+70S RNA + 70S(εA) ^c	0.07	0
+70S RNA·dT ₁₂₋₁₈	42.8	690
+70S RNA·dT ₁₂₋₁₈ + 70S(εA) ^c	34.2	552
B. DNA polymerase (<i>M. luteus</i>)		
-Template	0.05	1
+d(AT) ¹	5.1	14.1
+d(AT) ²	36.2	100
+d(AT) ¹ + 70S(εA) ^c	6.2	17.1
+d(AT) ² + 70S(εA) ^c	36.8	102
C. RNA polymerase (<i>E. coli</i>)		
-Template	0.03	1
+d(AT) ¹	3.2	10.8
+d(AT) ²	29.7	100
+d(AT) ¹ + 70S(εA) ^c	3.6	12.1
+d(AT) ² + 70S(εA) ^c	29.1	100

^a Reaction conditions for assaying the polymerase are those indicated in Materials and Methods. For the AMV enzyme the concentrations of 70S RNA and 70S(εA) where not indicated were 0.4 A₂₆₀ units per ml. For the two bacterial polymerases d(AT) was used as the template at two different concentrations of 0.04 A₂₆₀ units ml⁻¹ and 0.4 A₂₆₀/ml². Amount of purified AMV polymerase used was 5 μg per assay. 70S RNA not preincubated at 37 C concentration added 2.4 A₂₆₀ units per ml.

^b 70S RNA preincubated at 37 C for 1.5 h in the absence of chloroacetaldehyde concentration added 2.4 A₂₆₀ units per ml.

^c 70S(εA)RNA after 1.5 h of modification concentration added 2.4 A₂₆₀ units per ml.

value in identifying important bases involved in the recognition process. In this paper we have presented a novel chemical modification of AMV 70S RNA, which under controlled chemical conditions result in the specific modification of adenine residues.

Our initial interest in these studies stemmed from reports that a poly(A) region at the 3'-terminal end was present in oncogenic viral RNAs and is implicated in a variety of functions (8,

14). Modification of 70S RNA by treatment with chloroacetaldehyde is relatively gentle since it requires incubation at 37 C in aqueous solution and mild pH conditions. An additional advantage of this modification is the volatile nature of the reagent.

The number of residues modified in 70S RNA cannot be correlated with the observed increase in fluorescence quantum yield, since the quantum yield for a modified residue incorporated into a polynucleotide differs from the monomer. Lherach and Scheit (11) have reported that the quantum yield for poly(εA) was 0.024, whereas for εADP it was 0.6. These latter results show that interaction with neighboring bases, or other constraints imposed by the polynucleotide structure, quenches the fluorescence of ε-adenine. The net result is that residues in different microenvironments will have their fluorescence quenched to different degrees. Thus, the observed increase in fluorescence yield may not be a linear function of the number of bases modified. The exact number of modified residues must await digestion and analysis of the RNA. Such experiments are underway.

Transcription of 70S AMV RNA by AMV polymerase requires 4S RNA natural primers. The loss of template activity in 70S(εA)RNA (Fig. 6A, Table 1A) can be the result of the modification of the small RNA molecules or alternatively due to the modification on the 70S RNA template. The procedure for 70S RNA modification we have described appears to ef-

TABLE 2. *Additions of intact or chemically modified components to 70S-directed DNA synthesis by AMV DNA polymerase*

Components added to assay mixture ^a	Amount of [³ H]dTTP incorporated (pmol/30 min)	Activity (%)
70S RNA	6.58	100
70S RNA + 70S(εA)RNA	0.36	5
70S RNA + rA	4.85	74
70S RNA + rA(εA)	1.49	23
70S RNA + tRNA	5.19	80
70S RNA + tRNA(εA)	5.32	81
70S RNA·dT ₁₂₋₁₈	64.6	100
70S RNA·dT ₁₂₋₁₈ + 70S(εA)RNA	48.5	90
70S RNA·dT ₁₂₋₁₈ + rA	28.4	79
70S RNA·dT ₁₂₋₁₈ + rA(εA)	30.1	80

^a Assays were performed as described in Table 1. Each assay (100 μliters) contained 0.041 A₂₆₀ units of 70S RNA. Concentration of other nucleic acids added was the same in all experiments (0.24 A₂₆₀ units).

FIG. 6. Relationship between the extent of modification of 70S RNA on its template and inhibitory activity for AMV DNA polymerase. 70S RNA (2.9 O.D./ml) was incubated with chloroacetaldehyde at 37 C in a total volume of 1 ml. A parallel control experiment using the same amount of 70S RNA was performed in the absence of chloroacetaldehyde. At the indicated time intervals 100- μ l samples from both control and chloroacetaldehyde treated 70S RNA were removed, lyophilized to remove chloroacetaldehyde, and residues were suspended in 50 μ l of water. For template activity samples (5 μ l) from lyophilized material were added to assay mixture (50 μ l) of AMV DNA polymerase in the presence or absence of oligo(dT). For inhibitory activity samples (10 μ l) were added to reaction mixture containing either 70S RNA (0.06 A_{260} units per ml) or 70S RNA dT₁₂₋₁₈ [0.06 A_{260} units per ml and 0.013 A_{260} units per ml of oligo(dT)]. Amount of enzyme used in each assay was 5 μ g. (A) Template activity of 70S (ϵ A) RNA on AMV DNA polymerase. Symbols: ●, fluorescent intensity (arbitrary unit); ■, 70S RNA dT₁₂₋₁₈ incubated at 37 C in the absence of chloroacetaldehyde; Δ, 70S RNA incubated at 37 C in the absence of chloroacetaldehyde; ▲, 70S (ϵ A); □, 70S (ϵ A) dT₁₂₋₁₈. (B) Inhibitory activity of 70S (ϵ A) RNA on AMV DNA polymerase. ⊗, 70S RNA dT₁₂₋₁₈ + 70S (ϵ A) RNA, □, 70S RNA + 70S (ϵ A) RNA; ●, fluorescent intensity (arbitrary unit).

fect the template since the fluorescence observed was found associated with the 35S RNA subunit (Fig. 5). A second method of 70S RNA initiation may be achieved by priming 70S RNA with oligo(dT). The site for such initiation is believed to be the poly(rA) region identified at the 3' end of the 70S RNA molecule (8). The loss of activity upon oligo(dT) priming which we have observed is probably due to ϵ A formation in that region, resulting in the loss of base pair formation. Such an effect was also corroborated upon modifying poly(rA) (J. G. Chirikjian and T. S. Papas, *Biochem. Biophys. Res. Commun.*, in press). However, the nature of the reaction is such that internal adenosine modification also occurs which may well contribute to conformational changes in the RNA molecule.

There are several important questions in connection with these 70S RNA fragments which are currently under investigation in our respective laboratories. To date, we have initiated studies to fractionate fragments of 70S

RNA derived from extensive modification, and to characterize their size and composition. Secondly, fluorescent probes are being exploited in studies dealing with the effect of the chemical environment on the conformation of 70S RNA in solution and interaction of the fluorescent 70S RNA with the purified polymerase.

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