Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part I, pp. 3400–3404, December 1973

Reticulocyte RNA-Dependent RNA Polymerase

(RNA replicase/hemoglobin mRNA/heme)

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Communicated by Charles H. Rammelkamp, July 20, 1973

ABSTRACT **A cytoplasmic, microsomal bound RNA-dependent RNA polymerase has been purified 2500-fold from rabbit reticulocyte lysates. The synthesis of RNA with the purified enzyme is absolutely dependent on the addition of an RNA template. The best template is hemoglobin messenger RNA, while bacteriophage RNA and poly(A,G) are less active, and DNA is completely inactive as a template. With poly(A,G) as a template, only UTP and CTP are incorporated into polynucleotide chains, indicating that the RNA polymerase is an RNA replicase and not a terminal transferase. With messenger RNA as a template, all four ribonucleoside triphosphates are required for maximal activity. The RNA-dependent RNA polymerase reaction is extremely sensitive to low concentrations of heme, rifamycin AF/013, and ribonuclease and resistant to actinomycin D and DNase. The discovery of RNA-directed RNA synthesis in reticulocytes offers an additional site for control of gene expression in mammalian cells and provides a possible mechanism for amplification of the expression of specific genes.**

The control of gene expression in mammalian cells is usually discussed in terms of transcriptional control in the nucleus and translational control in the cytoplasm. Translational control mechanisms are thought to be of particular importance in higher organisms because of the presumed metabolic stability of mRNA in differentiated mammalian cells. This is based in part on the observation that reticulocytes, the immature erythrocytes which are anucleate and therefore not capable of synthesizing RNA, still actively synthesize hemoglobin (1, 2). The persistence of or increase in the synthesis of many enzymes after the administration of inhibitors of DNA-dependent RNA synthesis, such as actinomycin D, has also been cited as evidence that mRNA is stable in differentiated mammalian cells (see refs. 3–5, for reviews). However, the data are equally compatible with the hypothesis that mRNA is continually synthesized in the presence of actinomycin D but from an RNA rather than a DNA template.

In this communication we wish to report the partial purification and characterization of an RNA polymerase from rabbit reticulocytes that uses mRNA as a template for RNA synthesis. Although RNA replicases have been demonstrated in RNA viruses (6, 7), no RNA polymerase that uses an RNA template has been reported in a mammalian system. The demonstration of an RNA replicase in the microsomal fraction of an anucleate cell would establish an additional step in the flow of genetic information and an additional site for the control of gene expression in the cytoplasm of higher organisms.

Abbreviation: TSED buffer, 50 mM Tris·HCl (pH 7.8)–1.0 mM dithiothreitol–1.0 mM EDTA–0.25 M sucrose.

MATERIALS AND METHODS

[^{3}H]UTP and [^{3}H]CTP were purchased from either Schwartz-Mann or International Chemical and Nuclear Corp. Unlabeled ribonucleoside triphosphates were purchased from Sigma Chemical Co. Calf-thymus DNA, DNase 1, and RNase A were obtained from Worthington Biochemical Corp. Chicken erythrocyte DNA, bone-marrow DNA, actinomycin D, actidione, rifampicin B, heme, hemin, and RNase T1 were obtained from Calbiochem. Qβ RNA, MS2 RNA, poly(A), poly(U), poly(A,G) (2:1), poly(A)·poly(U), and poly(rA)·poly(dT) were purchased from Miles Chemical Co.

T4 DNA was prepared as by Bolle *et al.* (8). Rifamycin AF/013 was a gift of Drs. G. Lancini and R. Criechio of Lepetit, Milan and α-amanitin was a gift of Dr. T. Wieland of the Max Planck Institute, Heidelberg.

Preparation of RNA-Dependent RNA Polymerase. Reticulocytosis was induced in rabbits by a modification of the method of Borsook (9). New Zealand rabbits weighing 4–6 lb (1.8–2.7 kg) were given daily injections of 1.0 ml of a neutralized 2.5% phenylhydrazine solution for 4 days. The rabbits received no injections on the fifth and sixth days and were bled by cardiac puncture on the seventh day. Whole blood contained more than 80% reticulocytes.

Washed reticulocytes were prepared by repeated suspension of the cells in 0.13 M NaCl–5.0 mM KCl–7.4 mM $MgCl_2$, centrifugation at 2000 × *g*, and removal of the buffy coat. Washed reticulocyte preparations usually contained 0.5% nycleated cells of which about 50% were immature reticulocytes.

The reticulocytes from 400 ml of whole blood were lysed by the addition of 250 ml of lysis buffer [10 mM Tris·HCl (pH 7.4)–15 mM KCl–5 mM 2-mercaptoethanol] followed by gentle stirring for 20 min at 0°. Mitochondria and cell debris were removed by centrifugation at 30,000 × *g* for 15 min. The 30,000 × *g* supernatant was made 5.0 mM in $MgCl_2$ and the polysome fraction was obtained by centrifugation at 78,000 × *g* for 120 min. The ribosomal pellets were rinsed with lysis buffer and suspended in TSED buffer [50 mM Tris·HCl (pH 7.8)–1.0 mM dithiothreitol–1.0 mM EDTA–0.25 M sucrose] containing 0.5 M KCl by gentle homogenization in a glass homogenizer. The suspension was stirred for 30 min at 0°, then centrifuged at 152,000 × *g* for 60 min. The 152,000 × *g* supernatant, containing the solubilized polymerase activity, was brought to 60% saturation with ammonium sulfate and left overnight at 0°. The ammonium sulfate precipitate was collected by centrifugation at 30,000 × *g* for 15 min, dissolved in 6 ml of TSED buffer, and dialyzed against 1 liter of TSED

buffer for 3 hr with one change of buffer. The dialyzed extract was applied to a phosphocellulose column (2.3 × 9 cm), previously equilibrated with TSED buffer containing 0.05 M KCl, and washed with the same buffer. A linear gradient of 0.05–1.0 M KCl in TSED buffer with a combined volume of 300 ml was applied, and 3-ml fractions were collected. RNA-dependent RNA polymerase activity was eluted in a single peak at 0.45 M KCl (Fig. 1).

A summary of the enzyme purification is shown in Table 1. This procedure resulted in about 2500-fold purification of the enzyme as compared to the 30,000 × *g* supernatant fraction. The yield of enzyme activity was about 20%. Although the addition of mRNA stimulated the incorporation of [^{3}H]UTP at all stages of purification, an absolute requirement for added template could only be demonstated after phosphocellulose chromatography. The most purified enzyme preparations were very unstable, and most of the activity was lost in 3–4 days when the enzyme was stored in 0.25 M sucrose at 0°. However, when the enzyme was stored in 50% glycerol at −70°, it retained 50% of its original activity after 3 months.

To rule out the possibility that the RNA-dependent RNA polymerase was being isolated from the small number of leukocytes still remaining in the washed reticulocyte preparations, the enzyme purification procedure was carried out on washed leukocytes prepared from equal numbers of nonanemic rabbits. No RNA-dependent RNA synthesis was detected in this preparation.

Preparation of RNA Templates. RNA was prepared from the 78,000 × *g* ribosomal pellet by the method of Oda and Joklik (10) and fractionated by sucrose density gradient sedimentation (11). The template activity of each fraction was tested. Those fractions containing the highest activity (6–12 S + 16–20 S) were pooled, lyophilized, dissolved in 15 mM NaCl–1.5 mM Na citrate, and dialyzed. Although the 4S and 28S RNA fractions had some template activity, the greatest activity was seen with those fractions sedimenting in the range of hemoglobin mRNA (6–12 S) and 18S ribosomal RNA (16–20 S).

Assay for RNA-Dependent RNA Polymerase. The reaction mixture contained in a final volume of 0.25 ml: 80 mM Tris·HCl (pH 7.8); 1.6 mM $MnCl_2$; 1.0 mM EDTA; 1.0 mM dithiothreitol; 80 mM ammonium sulfate; 0.16 mM each ATP, GTP, and CTP; 8.0 μM [^{3}H]UTP, 250 Ci/mol; 5 μg of mRNA; and 10–15 μg of enzyme. After incubation for 30 min at 37°, the reaction was stopped by the addition of 2 ml of cold 5% trichloroacetic acid and the precipitate was collected on a glass-fiber filter (Whatman GF/C). The filter was washed with 30 ml of 5% trichloroacetic acid and 10 ml of 95% ethanol, dried, and counted in a liquid scintillation spectrometer.

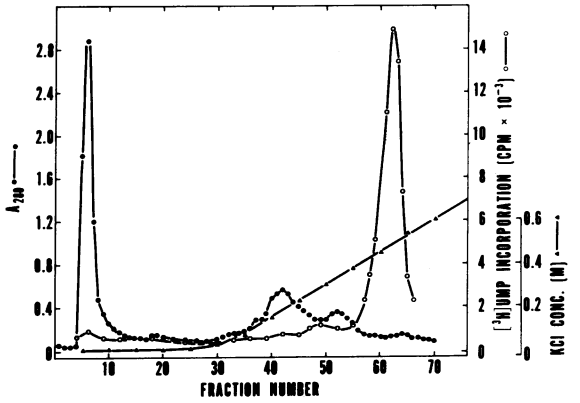

FIG. 1. Phosphocellulose chromatography of dialyzed ammonium sulfate fraction. 6.0 ml of extract (8.5 mg/ml) was applied to a 2.3 × 9-cm column. A linear gradient of 0.05–1.0 M KCl in TSED butter was run. The RNA polymerase activity was eluted in a single peak at 0.45 M KCl. ●——●, A_{280}; ○——○, enzyme activity; ▲——▲, KCl concentration. The numbers on the right-hand ordinate have been multiplied by 10^{-3}.

RESULTS

Requirements for RNA-dependent RNA synthesis

The RNA-directed synthesis of RNA shows an absolute requirement for a template and a divalent cation (Table 2). The rate of RNA synthesis is stimulated over 2-fold by the addition of either a sulfhydryl reagent or monovalent cation.

An absolute requirement for all four ribonucleoside triphosphates could not be demonstrated, although the rate of incorporation of [^{3}H]UTP is markedly stimulated by the addition of the other three ribonucleoside triphosphates. Consider-

TABLE 1. *Purification of RNA-dependent RNA polymerase*

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Fold purification	% Yield
30,000 × *g* supernatant	1920	21,670	0.09	—	100
Ribosomal homogenate	1680	260	6.5	72	87
176,000 × *g* supernatant	773	96	8.0	88	41
60% $(NH_4)_2SO_4$ precipitate	1000	51	19.6	215	51
Phosphocellulose chromatography	364	1.64	225.0	2500	19

Assay conditions were as described in *Methods*. A unit of polymerase activity is defined as that amount of enzyme which incorporated 1 pmol of UMP in 30 min of incubation at 37°.

TABLE 2. *Requirements for RNA-dependent RNA synthesis*

Reaction conditions	[^{3}H]UMP incorporated (pmol)	% Control
Complete	20.0	100
− dithiothreitol	6.9	35
− divalent cation	0.8	4
− monovalent cation	10.1	51
− template	0.6	3
− enzyme	—	—
− ATP, GTP, CTP	4.4	22

Incubation conditions were as described in *Methods* except that the individual components of the reaction mixture were omitted as indicated. Hemoglobin mRNA (6–12S RNA) was used as template.

TABLE 3. *Ribonucleoside triphosphate requirement for RNA synthesis with poly(A,G) (2:1) as template*

Ribonucleoside triphosphate present	[^{3}H]Nucleotide incorporated(cpm)
[^{3}H]UTP	870
[^{3}H]UTP,CTP	3110
[^{3}H]CTP	460
[^{3}H]CTP,UTP	910
[^{3}H]ATP	—
[^{3}H]ATP,GTP	—
[^{3}H]GTP	—
[^{3}H]GTP,ATP	—

Assay conditions were as described in *Methods* except (*i*) poly(A,G) (2:1) was used as template instead of mRNA, (*ii*) [^{3}H]ribonucleoside triphosphates and unlabeled ribonucleoside triphosphates were added as indicated, and (*iii*) no ammonium sulfate was added.

able amounts of DNA synthesis in the absence of one or more deoxyribonucleoside triphosphate has also been observed with several mammalian DNA polymerases (12–14).

Since an abolute requirement for all four ribonucleoside triphosphates cannot be demonstrated, it is important to determine whether the synthesis of RNA requires an RNA template or an RNA primer. We therefore examined the ribonucleoside triphosphate requirement for polynucleotide synthesis in the presence of poly(A,G) (2:1) (Table 3). It can be seen that the complementary ribonucleoside triphosphates [^{3}H]UTP and [^{3}H]CTP are both incorporated into an acid-precipitable product, while neither [^{3}H]ATP nor [^{3}H]GTP are incorporated. Furthermore, the presence of CTP stimulates the incorporation of [^{3}H]UTP and the presence of UTP stimulates the incorporation of [^{3}H]CTP. These results are consistent with the template-directed synthesis of a polynucleotide, in which the RNA product is transcribed from a complementary template, and not with an RNA-primed reaction, where ribonucleoside triphosphates are added to an existing primer, and suggest that the enzyme is an RNA replicase and not a terminal transferase.

Divalent Cation Requirement. The effect of divalent cations on the rate of RNA synthesis is shown in Fig. 2. $MnCl_2$ at its optimal concentration (1.4–1.6 mM) best satisfies the requirement for a divalent cation, although a low rate of RNA synthesis is seen at much higher concentrations of $MgCl_2$ (8.0–10.0 mM). The rate of RNA synthesis at the optimal $MnCl_2$ concentration is 9 times that with $MgCl_2$.

TABLE 4. *Template specificity of RNA-dependent RNA polymerase*

Template	Conc. (μg/assay)	[^{3}H]UMP incorporated (pmol)
None	—	—
6–12S RNA	5	14.4
16–20S RNA	5	14.9
Qβ RNA	4.5	3.5
MS2 RNA	4.5	2.0
T4 DNA	9	0.3
Calf-thymus DNA	10	—
Bone-marrow DNA	5	—
Chicken erythrocyte DNA	10	0.27
poly d(A-T)	4	—
poly(rA)·poly(dT)	5	—
poly(A)·poly(U)	10	—
poly(A)	5	0.5
poly(U)	5	—
poly(A,G) (2:1)	5	2.1

Reaction conditions were as described in *Methods* except for the addition of templates as indicated.

Effect of Monovalent Cations. The stimulation of the rate of RNA synthesis by monovalent cations is shown in Fig. 3. NH_4^+, K^+, and Na^+ all stimulate the reaction, and the optimal concentration for each is about 0.2 M. Both NH_4Cl and $(NH_4)_2SO_4$ stimulate the reaction to the same extent, and, as expected, the optimal concentration of $(NH_4)_2SO_4$ is half that of NH_4Cl. K^+ and Na^+ are less effective in stimulating the rate of RNA synthesis, suggesting that the stimulatory effect is not solely a function of ionic strength.

Time Course of the Reaction. The synthesis of RNA continues for at least 2 hr (Fig. 4). The rate is linear for at least 30 min and slowly declines after that.

Template Specificity. The ability of several RNAs, DNAs, and synthetic polynucleotides to serve as templates for the synthesis of RNA is shown in Table 4. No DNA was found to have any template activity with this enzyme. Of the synthetic polynucleotides tested, only poly(A,G) (2:1) was

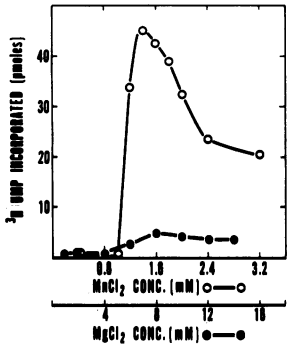

FIG. 2. Effect of divalent cations. Assay conditions were as in *Methods* except for the concentration of divalent cation. ●——●, $MgCl_2$; ○——○, $MnCl_2$.

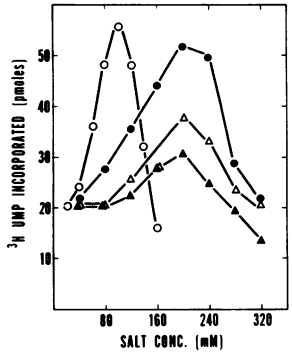

FIG. 3. Effect of monovalent cations. Assay conditions were as in *Methods* except for the salt concentration. ○——○, $(NH_4)_2SO_4$; ●——●, NH_4Cl; △——△, KCl; ▲——▲, NaCl.

found to have some template activity. The bacteriophage RNAs, MS2, and Qβ, were relatively poor templates, being about as active as poly(A,G).

The most active templates for this enzyme are the 6–12S RNA (mRNA), and 16–20S RNA (rRNA) isolated from reticulocyte polysomes. The template activity of the RNA sedimenting in the range of 18 S may be due to the presence of mRNA species sedimenting at this S value rather than 18S rRNA. A cytoplasmic precursor of 9S hemoglobin mRNA has been shown to sediment at 17 S (15).

Inhibitors of RNA Synthesis. The RNA-directed synthesis of RNA is completely resistant to actinomycin D and DNase either with the purified enzyme and an exogenous RNA template or with crude enzyme preparations and endogenous template (Table 5). The enzyme is also insensitive to α-amanitin and rifampicin. The former has been shown to be a potent inhibitor of eukaryotic nucleoplasmic DNA-dependent RNA polymerases (16, 17) and the latter an inhibitor of both bacterial and mitochondrial DNA-dependent RNA polymerases (18, 19). The RNA-dependent RNA polymerase is markedly inhibited by rifamycin AF/013. This antibiotic has been shown to inhibit eukaryotic DNA-dependent RNA polymerases (20–22), cytoplasmic DNA polymerase from bone marrow (23), and viral RNA-dependent DNA polymerase (24) and, thus, appears to be a general inhibitor of both DNA and RNA polymerases.

The synthesis of RNA with hemoglobin mRNA as template is not affected by known inhibitors of hemoglobin synthesis at the translational level such as actidione and NaF (25). However, in contrast to the translation of hemoglobin mRNA which is stimulated by heme, this compound markedly inhibits the RNA-directed synthesis of RNA.

Low concentrations of either RNase A or T1 completely inhibit the synthesis of RNA. This may be due to degradation of either the template or the product. The same result is obtained when RNase is added at the end of the incubation period, both at low and high ionic strength. This result would suggest that the product is probably single-stranded RNA.

DISCUSSION

The discovery of RNA-directed RNA synthesis in reticulocytes has important biological implications. It offers an additional site for control of gene expression in mammalian cells as well as providing a mechanism for amplification of the expression of specific genes.

Gene amplification, or the production of multiple gene copies, has been shown to occur in the nucleoli of amphibian oocytes for the genes for ribosomal RNA (26–28). Presumably this is the mechanism by which these cells are able to synthesize large quantities of rRNA in a relatively short time. A similar mechanism has been postulated to account for the rapid rate of synthesis of hemoglobin in erythroid cells. However, DNA·RNA hybridization studies with globin mRNA have shown that there is little or no reiteration of the globin genes in duck-erythrocyte nuclei (29), and no specific gene amplification was detected in immature duck erythrocytes. The amplification of mRNA by a cytoplasmic RNA-dependent RNA polymerase would allow for a large increase in the rate of synthesis of specific proteins without production of multiple gene copies.

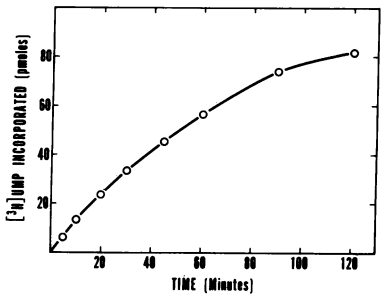

FIG. 4. Time course of the reaction. Assay conditions were as reported in *Methods* except for the time of incubation.

TABLE 5. *Effects of inhibitors on RNA-dependent RNA synthesis*

Inhibitor	[³H]UMP incorporated (pmol)	% Inhibition
Control	49.4	—
Actinomycin D (20 μg/ml)	49.4	0
α-Amanitin (8 μg/ml)	45.7	7
Rifampicin (20 μg/ml)	44.2	11
Rifamycin AF/013 (16 μg/ml)	1.8	96
DNase (10 μg/ml)	48.5	1
RNase A (5 μg/ml)	—	100
RNase T1 (5 units/ml)	0.3	99
Actidione (40 μg/ml)	49.7	0
NaF (16 mM)	41.8	15
Heme (4.0 μM)	18.8	62
(20 μM)	1.5	97

Reaction conditions were as described in *Methods* except for the addition of inhibitors as indicated.

The fact that only ribonucleoside triphosphates that are complementary to a synthetic polynucleotide template are incorporated into the RNA product and that all four ribonucleoside triphosphates are required for maximal activity with mRNA as a template indicates that the reticulocyte RNA-dependent RNA polymerase is an RNA replicase and not a terminal transferase. The reaction is not inhibited by inhibitors of DNA-dependent RNA synthesis, actinomycin D and α-amanitin; however, it is markedly inhibited by rifamycin AF/013. The observation that the RNA-dependent RNA polymerase reaction is not inhibited by actinomycin D might explain the lack of effect of actinomycin D on the synthesis of hemoglobin in immature erythroid cells (30) as well as the phenomenon of superinduction of many inducible enzymes observed in the presence of this antibiotic in other mammalian tissues (31).

The inhibitory effect of heme on RNA-dependent RNA synthesis is of particular interest. Heme has been postulated to control globin synthesis in the reticulocyte, although the mechanism by which control is exerted is not clear. It is known, however, that in reticulocytes and reticulocyte lysates the rate of globin synthesis decreases markedly and polysomes disaggregate after a few minutes unless heme is present (32, 33). Furthermore, these effects are reversible by the later addition of heme. The concentration of heme that results in complete inhibition of RNA synthesis (10 μM) is the same as that which results in optimal stimulation of protein synthesis (34). Recently heme has been shown to affect the translation of

other proteins in mammalian systems, both erythroid and nonerythroid (35).

In the immature erythrocyte, globin mRNA would serve both as a template for the RNA-dependent RNA polymerase and as a mRNA for protein synthesis. Thus, a competition would exist between replication and translation of the mRNA. The accumulation of heme in the maturing reticulocyte might be instrumental in allowing the preferential translation of mRNA by inhibiting the RNA-dependent RNA polymerase.

In the replication of RNA phage, an analogous situation exists, i.e., a single-stranded RNA must serve both as template for RNA replication and as a mRNA for protein synthesis. It has been suggested that the binding of Qβ replicase to RNA prevents the attachment of ribosomes and thus inhibits protein synthesis (36). The binding of RNA-dependent RNA polymerase to hemoglobin mRNA may prevent the translation of mRNA in the reticulocyte by a similar mechanism.

The translation of globin mRNA has also been shown to be extremely sensitive to the presence of double-stranded RNA (37). Recently Kaempfer and Kaufman have shown that the inhibition of protein synthesis by double-stranded RNA is due to inhibition of initiation factor 3, which is tightly bound by double-stranded RNA (38). The mechanism of RNA-dependent RNA synthesis is not understood. However, whether a double-stranded replicative form is first synthesized as a template for synthesis of identical strands of mRNA, or whether the RNA product synthesized is complementary to the RNA template, double-stranded RNA would be present in the reticulocyte during the synthetic process. Thus, the presence of double-stranded RNA as an intermediate in RNA replication would allow preferential transcription of RNA, even in the presence of ribosomes, since initiation of translation would be inhibited. At later stages of maturation of the erythrocyte, when the presence of heme had inhibited the replicase, no double-stranded RNA would be produced to interfere with protein synthesis.

We thank Dr. William J. Harrington for continuing interest and support. This research was supported by grants from the National Institutes of Health (NIH AM 09001 and NIH-5-T01-AM-5472-03), American Heart Association (72-967), and Milton N. Weir, Jr. Cancer Research Fund. A.G.S. holds an Established Investigatorship from the American Heart Association.

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