***In Vitro* RNA-dependent RNA Synthesis by a Curde Enzyme Fraction Isolated from Normal Rat Livers**

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ABSTRACT. A crude enzyme preparation from “uninfected” rat livers can catalyze the synthesis of RNA when rat liver mRNA or nuclear RNA is added to the system. The newly synthesized RNA contains sequences complementary to those of the RNA template and, after phenol extraction, is found to be partially hydrogen-bonded to the added poly (A)-containing RNA. The possibility of message amplification in normal cells is discussed.

The belief to date is largely that in higher organisms, RNA is synthesized only on a DNA template by a DNA-dependent RNA polymerase, exceptions being RNA virus-infected cells, where it has been shown that a viral RNA-dependent RNA polymerase is responsible for viral RNA synthesis (1, 2). However, the possibility of RNA-dependent RNA synthesis being involved in normal cellular function has been described (3, 4). Recently an enzyme fraction, crude or partially purified from “uninfected” animal and plant tissues, was reported, which catalyzed the *in vitro* synthesis of RNA after addition of RNA but not DNA (5–9).

In this communication I describe an *in vitro* RNA-dependent RNA synthesis by a crude enzyme fraction isolated from “uninfected” rat livers. The result suggests that the enzyme product after phenol extraction was in part hydrogen-bonded to poly (A)-containing RNA which had been added to the reaction mixture and which was probably acting as a template.

MATERIALS AND METHODS

Materials. [5, 6-³H] Uridine-5'-triphosphate (52 Ci/mmole) and [α -³²P] uridine-5'-triphosphate (1.1 Ci/mmole) were purchased from the Radiochemical Centre, Amersham. Ribonuclease T₁, pancreatic ribonuclease A, deoxyribonuclease and oligo (dT)-cellulose were the same as those used in the previous publication (10). Poly (A), poly (U), poly (C) and poly (U, C) were from Miles Co. Rifampicin (Ciba Ltd.) and α -amanitin were donated by my colleague, Dr. P. Goodwin. Male Wistar rats (150–188 gm body weight) were used.

Preparation of RNA's. Unlabelled nuclear RNA (nRNA), double-stranded RNA (dsRNA), 28S rRNA and tRNA were prepared from rat livers by the procedures described previously (10, 11). Unlabelled mRNA was purified from rat liver polysomal RNA by sucrose density gradient centrifugation (10). Reovirus RNA was the same preparation as used in the previous publication (11).

Preparation of enzyme fraction. An enzyme fraction was prepared from rat livers by a modification of the method of Haruna *et al* (12). Fresh livers were passed through a mesh, suspended in Buffer A (100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.5 mM mercaptoethanol

and 5 $\mu\text{g/ml}$ deoxyribonuclease) and homogenized in a Waring blender after freezing and thawing. The post-mitochondrial supernatant obtained by centrifugation at 30,000 $\times g$ for 30 min was treated with 0.11 M EDTA for 5 min at 2°C and then clarified by centrifugation. The supernatant containing the solubilized enzyme was brought to 50% saturation with ammonium sulphate. The precipitate was collected by centrifugation at 17,000 $\times g$ for 10 min, dissolved in Buffer B (10 mM Tris-HCl, pH 7.4, 5 mM MgCl_2 , 0.5 mM mercaptoethanol, 0.4 M $(\text{NH}_4)_2\text{SO}_4$ and 20% (v/v) glycerol) and stored at -80°C .

Assay for enzyme activity. The standard reaction mixture contained in a final volume of 0.25 ml: 80 mM Tris-HCl, pH 7.9, 1.5 mM MgCl_2 , 0.6 mM MnCl_2 , 0.4 mM ATP, GTP and CTP, 0.08 mM labelled UTP (10 μC ^3H or approx. 20 μC ^{32}P -labelled UTP), 2.5 μg actinomycin D, unless otherwise indicated 65–67 μg nRNA, and 144–158 μg enzyme. After incubation at 36°C for 20 min (unless otherwise noted), the reaction was stopped by addition of pyrophosphate and trichloroacetic acid (12). The precipitate was collected on a glass-fiber filter and the radioactivity determined as described previously (11).

Preparation of enzyme product. After incubation in 5 to 10-fold standard reaction mixtures at 36°C for 20 min, labelled RNA was extracted by the sodium dodecyl sulphate-phenol procedure (13). The aqueous layer obtained after phenol extraction followed by centrifugation at 9,000 $\times g$ for 10 min was collected and dialyzed against 10 mM Tris-HCl, pH 7.6, 0.14 M NaCl for 18 hrs. The labelled material was precipitated with 2 volumes of absolute ethanol, washed with 80% ethanol several times, dissolved in 10 mM Tris-HCl or phosphate buffer, pH 7.4 and stored at -20°C . The labelled material obtained under these conditions—hereafter called “enzyme products”—contained both nRNA added to the reaction mixture and newly synthesized RNA containing labelled UMP.

Analysis of nucleoside and nucleotide content. Nucleotide content of ^{32}P -labelled enzyme product was analyzed by the method described previously (11). Labelled uridine and UMP content of ^3H -labelled enzyme product were determined by one dimensional chromatography on thin-layer cellulose plates using the development solution (66% isobutyric acid and 1% ammonia dissolved in distilled water).

RNA-RNA hybridization. Hybridization was carried out at 72°C for 24 hrs in 10 mM phosphate buffer, pH 7.6, 0.3 M NaCl. After annealing, the reaction mixtures were treated with ribonucleases and the radioactivity of the hybrids formed was determined as described previously (10).

Oligo (dT)-cellulose column chromatography. This was carried out by the method previously described (10). Non-poly (A) and poly (A)-containing RNA fractions of enzyme products were obtained by washing the column with 10 mM Tris-HCl, pH 7.6, 0.5 M NaCl and by eluting with 10 mM Tris-HCl, pH 7.6, respectively.

RESULTS AND DISCUSSION

Some properties of the incorporation systems. In the standard reaction mixture, ^3H -UTP was incorporated into the acid-insoluble fraction as shown in Fig. 1. Omission of nRNA from the reaction mixture abolished ^3H -UTP incorporation, indicating an almost absolute requirement for added RNA for the reaction. Removal of actinomycin D from the reaction mixture resulted in a slight (less than 10%) enhancement of ^3H -UTP incorporation. The reaction was optimal at pH 7.9 and showed a definite requirement for Mn^{++} . Maximum activity was seen at a concentration of 0.6 mM Mn^{++} in the presence of 1.5 mM Mg^{++} (Fig. 2).

The enzyme(s) showed some characteristics expected of an RNA-dependent RNA polymerase (2) or a similar type of enzyme (Table 1). The reaction was not significantly affected by orthophosphate, α -amanitin, rifampicin or deoxyribonuclease,

Fig. 1. The effect of added nRNA on the kinetics of ^3H -UTP incorporation. The composition of the standard reaction mixture was as in the text with the following exception. $\bigcirc$ — $\bigcirc$, with $216 \mu\text{g/ml}$ nRNA and $10 \mu\text{g/ml}$ actinomycin D.; $\bullet$ — $\bullet$, without nRNA; $\times$ — $\times$, with $216 \mu\text{g/ml}$ nRNA, but without actinomycin D.

Fig. 2. The effect of varying concentrations of Mg^{++} or Mn^{++} on ^3H -UTP incorporation. Assay conditions were as in the text except for the concentration of divalent cations and nRNA. $\bigcirc$ — $\bigcirc$, with or $\bigcirc$ — $\bigcirc$, without $268 \mu\text{g/ml}$ nRNA, but at varied concentration of Mn^{++} in the presence of 1.5 mM MgCl_2 ; $\bullet$ — $\bullet$, with or $\bullet$ — $\bullet$, without $268 \mu\text{g/ml}$ nRNA, but at varied concentration of Mg^{++} .

excluding any significant contribution of polynucleotide phosphorylase or DNA-dependent RNA polymerase. Enzyme activity was markedly inhibited by the addition of ribonuclease or inorganic pyrophosphate.

TABLE 1. THE EFFECTS OF INHIBITORS ON RNA-DEPENDENT RNA SYNTHESIS

Inhibitors	Relative incorporation
Control (no inhibitor)	100
+ Pyrophosphate (1.1 mM)	16
+ „ (8 mM)	2
+ Orthophosphate (1.2 mM)	97
+ „ (10 mM)	70
+ α -amanitin (0.4 μ g/ml)	100
+ „ (4.0 μ g/ml)	91
+ Rifampicin (4.0 μ g/ml)	79
+ Deoxyribonuclease (53 μ g/ml)	98
+ Ribonuclease A (12 μ g/ml)	2

The standard reaction mixtures were as described in the text except for the addition of inhibitors as indicated. The control value was 1801 cpm.

Stimulatory activities of RNA and DNA. The stimulatory activity of synthetic and natural polynucleotides varied considerably (Table 2). Neither native nor denatured

TABLE 2. STIMULATORY ACTIVITIES OF RNA AND DNA

Addition	Relative incorporation
Control (no RNA or DNA)	100
DNA, rat liver (undenatured, 40 μ g/ml)	101
„ (heat-denatured, 40 μ g/ml)	95
nRNA (40 μ g/ml)	421
tRNA (40 μ g/ml)	267
28S rRNA (39 μ g/ml)	240
dsRNA (53 μ g/ml)	93
Reovirus RNA (34 μ g/ml)	101
Poly (A)•Poly (U) (200 μ g/ml)	77
Poly (A) (100 μ g/ml)	108
Poly (U) (100 μ g/ml)	147
Poly (C) (100 μ g/ml)	85
Poly (U, C) (100 μ g/ml)	122

The standard reaction mixtures were as described in the text except for the addition or omission of RNA or DNA as indicated. The control values ranged from 328 to 443 cpm in several experiments.

rat liver DNA showed any stimulatory activity. Marked stimulation was observed when rat liver nRNA or mRNA was added to the reaction mixture. The stimulation was dose-dependent (Fig. 3). The preparation of rat liver mRNA used in this experiment was highly purified and had been shown to contain messages for liver cellular proteins (10). On the other hand, the nRNA preparation contained a variety of RNA species in addition to heterogeneous nuclear RNA (HnRNA). Therefore, no precise comparison of efficiency of stimulation can be made between mRNA and nRNA.

Fig. 3. The effect of varying amounts of added RNA on ^3H -UTP incorporation. Assay conditions were described in the text except for the amounts of mRNA ($\bigcirc$ --- $\bigcirc$) or nRNA ($\bullet$ --- $\bullet$).

28S rRNA and tRNA exhibited low stimulatory activities. No significant stimulation of ^3H -UTP incorporation was observed with any of the synthetic RNAs used. Under standard assay conditions, all dsRNAs were inactive. Since nRNA effected good stimulation, unless otherwise stated, nRNA was routinely used for further investigations.

Characterization of the enzyme product. Since the enzyme products are completely sensitive to 0.3 N KOH, this is indicative of RNA species which incorporated labelled UMP. Moreover, the majority (98.4%) of the product was found to be single-stranded RNA and only 1.6% was ribonuclease-resistant (Table 3). This resistance was abolished when the enzyme products were heat-denatured before ribonuclease treatment using conditions which were specifically established for isolation of dsRNA (11). This indicates that a small portion of the newly synthesized RNA molecules is part of double-stranded structures. It is not clear whether this complex represents an *in situ* ribonuclease-resistant structure or a self-annealed artefact which occurred during phenol extraction (14, 15). The possibility exists that complementary copies may be formed on the templates and after phenol extraction these copies are partially hydrogen-bonded to the template. This case is analogous to that of viral RNA replication (2) and was supported by the results obtained in several experiments.

Fig. 4. Hybridization of labelled enzyme products at varying inputs of RNA. Enzyme products were prepared from 5-fold standard reaction mixtures which contained labelled UTP with unlabelled ATP, GTP and CTP (●—●). Hybridization was carried out as described in the text.

TABLE 3. RIBONUCLEASE SENSITIVITY OF ENZYME PRODUCTS

Treatment	³ H-labelled product remaining	
	(cpm)	(%)
Control (no treatment)	6904	100
Ribonuclease treatment	122	1.6
Heat-denaturation, followed by ribonuclease treatment	20	0.3

³H-Labelled enzyme products were prepared as described in the text. The ribonuclease treatment was carried out with ribonuclease A (10 μ g/ml), and ribonuclease T₁ (1 μ g/ml) in 10 mM phosphate buffer, pH 7.6 and 0.3 M NaCl at 37°C for 30 min.

When the enzyme products were annealed at increasing RNA inputs, the ribonuclease-resistance of the products increased. At the highest value of mg/ml/hr studied, about 3.7% of the total enzyme product was hybridized; however, saturation had not been reached (Fig. 4). Higher values of mg/ml/hr were not obtained, since an annealing period of 24 hrs at 72°C was adopted to avoid degradation of RNA (10). It is significant that the increased ribonuclease-resistance upon annealing was obtained only when the enzyme products were prepared from reaction mixtures containing the four nucleotide triphosphates. This increase in ribonuclease-resistance observed after annealing is not due to intramolecular self-annealing of the labelled products, but represents the formation of hybrids between labelled products and the added nRNA, probably HnRNA, for the following reasons: 1) Since heat-denaturation of enzyme products followed by rapid cooling failed to increase ribonuclease-resistance, an intermolecular association of labelled products seems likely (e.g., ref. 16). 2) The enzyme products hydrogen-bonded to poly (A)-containing RNA were recovered by oligo (dT)-cellulose column chromatography, indicating that the products were hybridized to poly (A)-containing RNA. It seems unlikely that poly (A)-containing

RNA is newly synthesized during the *in vitro* incubation in sufficient amounts to form hybrids with the labelled enzyme product. Therefore, labelled material appears to be hybridized to poly (A)-containing RNA which was added to the reaction mixture. 3) The poly (A) segments of HnRNA molecules were not involved in the hybridization reaction with newly synthesized poly (U), because the treatment of enzyme products with ribonuclease T_1 before annealing completely abolished the increase in ribonuclease-resistance (Table 4).

TABLE 4. THE EFFECT OF RIBONUCLEASE TREATMENT ON HYBRIDIZATION OF ENZYME PRODUCTS

Ribonuclease treatment		^3H -labelled product remaining	
Before annealing (with ribonuclease T_1)	After annealing (with ribonucleases T_1 and A)	(cpm)	(%)
—	—	5166	100
—	+	86	1.7
+	—	840	16
+	+	0	0

^3H -Labelled enzyme products were prepared as described in the text and treated with ribonucleases before and/or after annealing, as indicated. Ribonuclease treatment was carried out as described in Table 3. Hybridization was at 5.0 mg/ml/hr.

No increase in ribonuclease-resistance after annealing was observed when the products were isolated from reaction mixtures without ATP, GTP and CTP (data not shown here).

Evidence was further obtained that sequences of RNA complementary to those of poly (A)-containing RNA were newly synthesized.

When labelled enzyme products were subjected to fractionation on oligo (dT)-cellulose, 13–36% of the products, depending upon the enzyme preparation used, were found to be associated with poly (A)-containing RNA (Table 5). Since, as

TABLE 5. FRACTIONATION OF ENZYME PRODUCTS BY OLIGO (dT)-CELLULOSE COLUMN CHROMATOGRAPHY

Expt. No.	Substrates in reaction mixture	Heat-denaturation	Fractionation (% of total)	
			Non-poly (A) fraction	Poly (A) fraction
1	^3H -UTP	—	95.6	4.4
2	^3H -UTP + ATP + GTP + CTP	—	64.0	36.0
	” ” ” ”	+	96.9	3.1

^3H -Labelled enzyme products were prepared from 5-fold standard reaction mixtures which contained ^3H -UTP, unlabelled ATP, GTP and CTP or ^3H -UTP only, as described in the text. The products were fractionated into “poly (A)” and “non-poly (A)” fractions by oligo (dT)-cellulose column chromatography, with or without prior heat-denaturation. See the text for additional details.

mentioned previously, 1.6% of the total enzyme product was ribonuclease-resistant, only a small portion of the newly synthesized molecule appeared to be hydrogen-bonded to poly (A)-containing RNA, the rest of the molecule remaining single-stranded.

The net increase, 3.4% in ribonuclease-resistance on annealing at 52 mg/ml/hr (see Fig. 4) represents the proportion of newly synthesized material which is complementary to the added RNA. Since, as will be described later, the labelled material associated with poly (A)-containing RNA, comprising 13–36% of the total enzyme product, is the result of a heteropolymer-synthesizing reaction, it seems that at least 10–26% of newly synthesized heteropolymers can be hybridized to poly (A)-containing RNA. In our hybridizations, saturation was not reached. Consequently these values would certainly increase on annealing enzyme products at higher values of mg/ml/hr. However, the low values obtained here may also in part be due to the complexity of the “template” rRNA added and of the enzyme products.

³H-UTP incorporation into the products which are associated with poly (A)-containing RNA showed an almost absolute requirement for all four ribonucleotide triphosphates. Omission of three ribonucleotide triphosphates (ATP, GTP and CTP) resulted in failure to find any labelled material hydrogen-bonded to poly (A)-containing RNA (Table 5). However, due to an apparent stimulation in homopolymer synthesis and/or terminal addition of UMP, this omission did not result in a significant reduction (approximately 20%) of the total incorporation into enzyme products (data not shown here). The majority of ³H-UMP incorporated in the presence of all four ribonucleotide triphosphates was located internally in the RNA molecule (Table 6). The experiment using α -³²P-UTP showed 50.9% G + C content in the product, a

TABLE 6. DISTRIBUTION OF RADIOACTIVITY IN TERMINAL AND INTERNAL POSITIONS OF THE ENZYME PRODUCT

Expt. No.	Substrates in reaction mixture	Fractions on oligo (dT)-cellulose	Fractions isolated after alkaline hydrolysis (% of total)	
			Nucleoside	Nucleotide
1	³ H-UTP	Non-poly (A) fraction	67.8	32.2
	„	Poly (A) fraction	26.4	73.6
2	³ H-UTP + ATP + GTP + CTP	Non-poly (A) fraction	62.4	37.6
	„ „ „ „	Poly (A) fraction	<1	>99

³H-Labelled enzyme products were prepared and fractionated as described in Table 5. After alkaline hydrolysis, the radioactivities of nucleoside and nucleotide were analyzed as described in the text.

value similar to that (50.6–51.3%) of rat liver HnRNA (17, 18) added to the reaction mixture. These results indicate that the labelled material associated with poly (A)-containing RNA represents newly synthesized heteropolymers which are probably RNA transcripts from a poly (A)-containing RNA, HnRNA. In contrast, the labelled material which is not associated with poly (A)-containing RNA has large amounts of terminally added UMP (Table 6) and shows low G + C content (23.4%). The incorporation into this fraction is thus mainly due to terminal addition of UMP, probably to rRNA and tRNA molecules present in the nuclear RNA preparation, and possibly to the formation of homopolymers.

Taking all of these observations into account, it seems likely that the crude enzyme preparation contains an enzyme(s) which catalyzes the synthesis of RNA transcripts complementary to sequences in poly (A)-containing RNA. In the present experiments, I only found complementary copies to added poly (A)-containing RNA. The homology between the sequence of the products and that of the added RNA was not investigated. Therefore, the question remains whether the enzyme(s) eventually catalyzes the synthesis of copies homologous to added templates. The preparation also exhibits terminal addition of UMP and/or homopolymer synthesis. Recently, the enzyme(s) responsible for RNA-dependent RNA synthesis were purified from crude rat liver preparations by phosphocellulose column chromatography, followed by glycerol gradient centrifugation (19). The results obtained in the preliminary experiments show that the enzymes responsible for heteropolymer synthesis, on the one part, and homopolymer synthesis (and/or terminal addition), on the other, appear to be interrelated.

The biological significance of the discovery of RNA-dependent RNA synthesis in "uninfected" cells should be mentioned here. The possibility of viral origin of the enzyme(s) has not yet been excluded. However, the variety of enzyme sources so far employed (3, 5-9) and the template specificity of the enzymes suggest that viral origin is unlikely.

An attractive hypothesis is that a cell possesses a message amplification system in which the RNA-dependent RNA polymerase plays a vital role (4). The finding of the enzyme(s) in "uninfected" rat liver cells indicates that this mechanism may be part of fundamental regulatory machinery of the cell, and not be restricted to enucleated cells (8). With respect to this hypothesis, high template activity of mRNA and nRNA is of particular interest. HnRNA contains a long poly (A) segment at the 3'-end and is thought to be a precursor of cytoplasmic mRNA (20, 21). The fact that only mRNA and nRNA are very active templates for RNA-dependent RNA synthesis is in accord with the concept of message amplification: only mRNA and messenger-like RNA (or possibly particular species of mRNA) may be selectively recognized and amplified. In this communication, however, it would be premature to discuss this hypothesis in detail. Further experiments are certainly needed for a better understanding of RNA-dependent RNA synthesis.

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