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Biochemical Genetics, Vol. 12, No. 2, 1974

RNA-Bound Reverse Transcriptase in *Escherichia coli* and *in Vitro* Synthesis of a Complementary DNA

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Received 8 Mar. 1974—Final 25 Apr. 1974

RNA-bound reverse transcriptase can be easily distinguished from RNA-free reverse transcriptase and DNA-dependent DNA polymerase after fractionation of extracts from Escherichia coli *on a DEAE-cellulose column. This enzyme is capable of synthesizing a DNA-like product in the absence of exogenously added template provided that all four deoxyribonucleoside 5′-triphosphates are present in the incubation mixture. Removal of RNA from the enzyme by RNase leads to a considerably decreased polymerizing activity. The activity can be restored under appropriate conditions either by RNA originating from the enzyme or by transforming RNA excreted by showdomycin-resistant* E. coli. *Enzyme-bound RNA has several characteristics already found for the transforming RNA. DNA synthesized by RNA-bound reverse transcriptase is complementary to the enzyme-bound RNA.*

KEY WORDS: reverse transcriptase; RNA; DNA; *Escherichia coli.*

INTRODUCTION[2]

The RNA-directed DNA polymerase (reverse transcriptase) found in a number of RNA viruses (Temin and Mizutani, 1970; Baltimore, 1970; Gallo, 1971; Spiegelman *et al.*, 1970) has been purified in several laboratories (Kacian *et al.*, 1971; Duesberg *et al.*, 1971; Hurwitz and Leis, 1972; Faras

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[2] Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Sho-R, showdomycine resistance; dNTP, deoxyribonucleoside 5′-triphosphate; dNDP, deoxyribonucleoside 5′-diphosphate; RNase, ribonuclease; DNase, deoxyribonuclease; TCA, trichloroacetic acid.

poly(rA)poly(dT) 10–12 and poly(rA) from Miles Laboratories, U.S.A. Rifampicin and rifamycin [AF/0.13 L.8543 3-formyl-rifamycin SV *O*-octylooxyne and AF/ABDP-*cis* L.9254 4(*N*)-benzyl-4(*N*)-dimethyl-1-2,6-dimethylrifampicin] were synthesized by Dr. R. Cricchio, Gruppo Lepetit, Milan, Italy. Actinomycin D and Pronase were obtained from Calbiochem; Difco extract from Difco, U.S.A.; glass filter GF/C from Whatman, W. R. Balstorn, Ltd., England. RNases were heated for 10 min at 100 C before use. DNase was free of RNase.

Bacterial Strains

Escherichia coli strain K12 Hfr (Hayes) wild type was cultured either in synthetic medium 63 (Pardee *et al.*, 1959) containing glucose or in Difco Bactopeptone medium with shaking at 37 C. Bacteria were collected by centrifugation after exponential growth, and the pellet was washed with 10^{-2} M tris-HCl buffer containing 10^{-2} M $MgCl_2$ and 6×10^{-2} M KCl, *p*H 7.65. Enzyme was purified from bacteria grown in Difco medium. Showdomycin-resistant "mutant" M_{500} (Sho-R) (Beljanski *et al.*, 1972) was used as the source of transforming RNA.

RNA Determination

RNA-bound reverse transcriptase was separated from the enzyme by phenol and then chloroform, precipitated by alcohol, and dialyzed against distilled water containing 0.2 M KCl. The amount of RNA was determined by ultraviolet absorption at 260 nm and by the orcinol reaction (Beljanski, 1949). The RNA preparation was also analyzed for DNA content by the diphenylamine reaction, or by radioactivity when the RNA was isolated from RNA-bound reverse transcriptase originating from bacteria cultured in the presence of thymidine-C^{14}.

Nucleotide Analysis of RNA Bound to Reverse Transcriptase

RNA (1 mg) was hydrolyzed with 0.3 M KOH at 37 C for 24 hr. The hydrolysate was neutralized with 4 N HCOOH and adsorbed on a Dowex column (1×2, 200–400 mesh). After the column had been washed with distilled water, nucleotides were gradually eluted with a linear gradient, H_2O–4 N HCOOH, and identified by the 280/260 nm absorption ratio. Absorption of HCOOH at 260 nm was subtracted from each nucleotide-containing fraction after titration with NaOH. The following extinction coefficients were used to calculate the amount of each nucleotide: A = 14, G = 12, C = 6,8, and U = 10.

(40 by 3 cm) prepared as described in the Whatman Laboratory Manual IE3 (1973). The column was washed with tris-HCl (*p*H 7.5) buffer alone (300 ml). Proteins were eluted with a linear gradient: 10^{-2} M tris-HCl (*p*H 7.4), 1000 ml—10^{-2} M tris-HCl (*p*H 7.5) plus 0.5 M KCl, 1000 ml. Fractions of 5 ml were collected (*p*H adjusted to 7.5), and each fraction was tested for the ability to polymerize the dNTP in the absence or in the presence of exogenously added template RNA or DNA. Fractions from peak I were progressively mixed with solid $(NH_4)_2SO_4$ to 50% of saturation (*p*H maintained at 7.4). The precipitate collected by centrifugation was dialyzed overnight (4 C) against the same buffer solution. The 280/260 nm ratio of dialyzed proteins was 0.70.

Step 4: Sephadex G200 Column. The dialyzed fraction from step 3 was layered on a Sephadex G200 column (30 by 2 cm) in 10^{-2} M tris-HCl buffer (*p*H 7.6). Fractions of 3 ml were collected. The proteins were eluted with 10^{-2} M tris-HCl buffer containing 10^{-4} M $MgCl_2$ and 6.10^{-3} M KCl (*p*H 7.60). RNA-bound reverse transcriptase activity appears in those fractions which immediately precede the bulk of proteins (fraction 20). RNA-bound reverse transcriptase after filtration on a Sephadex G200 column maintains its endogenous activity.

Enzyme Assay

The incubation medium of 0.2 ml contained 25 μM tris-HCl (*p*H 7.65), 2 μM $MgCl_2$, 5 nmoles of each dNTP, dGTP-H^3 (50,000 cpm), 0.05 ml of enzyme fraction of DEAE column eluates, and 2–4 μg of template RNA of bacterial origin or thymus DNA. When not specified, 0.02 ml of enzyme fraction containing 40 μg of ptoteins was used. After incubation at 36 C for 20 min, the radioactive product was precipitated with TCA (5% final concentration), washed on a glass filter, and dried. Radioactivity was measured in a Packard spectrometer.

RESULTS

Detection and Activity of RNA-Bound Reverse Transcriptase

Among fractions eluted from the DEAE-cellulose column, one can distinguish three peaks which possess the ability to polymerize the dNPTs into an acid-precipitable product (Fig. 1). Peak I exhibits a high endogenous activity. Addition of exogeneous transforming RNA from *E. coli* or thymus DNA is without effect. Peak II (RNA-free reverse transcriptase) functions only in the presence of exogenously added RNA template (transforming RNA) (Beljanski *et al.*, 1971*c*), while peak III (DNA-dependent DNA polymerase I) requires the presence of a DNA template.

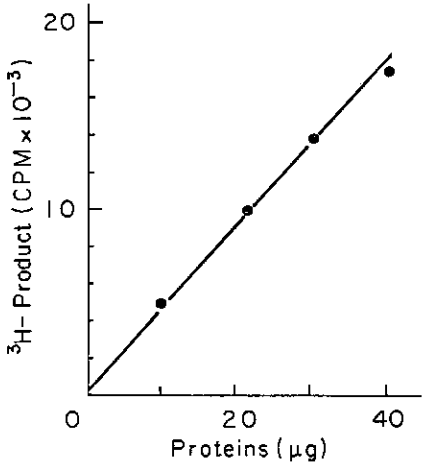

Fig. 2. Activity of RNA-bound reverse transcriptase used at different concentrations. For incubation mixture, see caption of Fig. 1 and text. Incubation time 20 min.

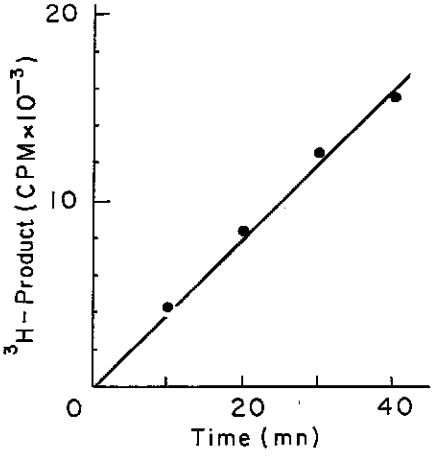

Fig. 3. Activity of RNA-bound reverse transcriptase as a function of time. RNA-bound reverse transcriptase (10 μg of protein) was incubated in the presence of 5 nmoles of each of the four dNTPs and 50,000 cpm of H^3-dGTP. Final volume 0.15 ml, temperature 36 C.

Precipitated and dialyzed protein fraction from peak I exhibits a high endogenous activity (Table I) only if all four dNTPs (dNDPs are inactive) are present in the incubation mixture. The activity increases as a function of enzyme concentration (Fig. 2). The amount of the H^3-product synthesized also increases as a function of time (Fig. 3). Heating of the enzyme for 10 min at 100 C completely abolishes the activity (Table I). Treatment of the enzyme with RNases (20 min at 24 C) leads to a considerably decreased amount of the TCA-precipitable H^3-product. However, some resistance of RNA to RNase A (less to RNase T_1) could be explained by the fact that this RNA is rich in purine nucleotides (G+A/C+U = 2.0; see Table VII). *Escherichia*

cellulose column. First, under the conditions described there is only one peak where DNA template is required for DNA polymerase I activity. Second, the endogenous activity of RNA-bound reverse transcriptase is not changed when exogenous RNA (transforming RNA) or DNA is added. Third, it is well known that rifampicin, rifamycin, and certain of their derivatives do not affect DNA polymerase activity (Table III) from *E. coli* at all (Riva and Silvestri, 1972), while these compounds do inhibit the activity of RNA-bound reverse transcriptase (Table II) to about 50–60% (the AF/0.13 compound is an exception). KCl at 0.2 M which almost completely inhibits the activity of DNA polymerases II and III and about 50% of that of RNA-bound reverse transcriptase, is without effect on DNA polymerase I (Table IV). *N*-Ethyl-

Table III. Inhibitors of RNA-Bound Reverse Transcriptase[a]

	pmoles of H^3-dGTP incorporated	Percent inhibition
Complete	336	—
+actinomycin D (2.5 μg)	150	56
+rifampicin (20 μg)	180	46
+rifamycin (20 μg)	168	50
+AF/ABDP-*cis* (20 μg)	172	49
+AF/0.13 (10 μg)	83	81
+AF/0.13 (20 μg)	30	90
+0.2 M KCl	170	51
+*N*-ethylmaleimide (10 min)	335	0

[a] Incubation conditions as in Materials and Methods section with 20 μg of RNA-bound reverse transcriptase. All compounds were added to the incubation mixture before enzyme.

Table IV. Activity of DNA-Dependent DNA Polymerase in the Presence of Various Compounds (inhibitors)[a]

	Cpm of H^3-dGTP incorporated	Percent inhibition
Complete	6340	—
+actinomycin D (2.5 μg)	2030	68
+rifampicin (20 μg)	6410	0
+rifamycin (20 μg)	6396	0
+AF/ABDP-*cis* (20 μg)	6500	0
+AF/0.13 (20 μg)	1130	82
+0.2 M KCl	6500	0
N-ethylmaleimide (10 mM)	6290	0

[a] Incubation conditions as in Materials and Methods section with 40 μg of DNA-dependent DNA polymerase (Fig. 1, peak III).

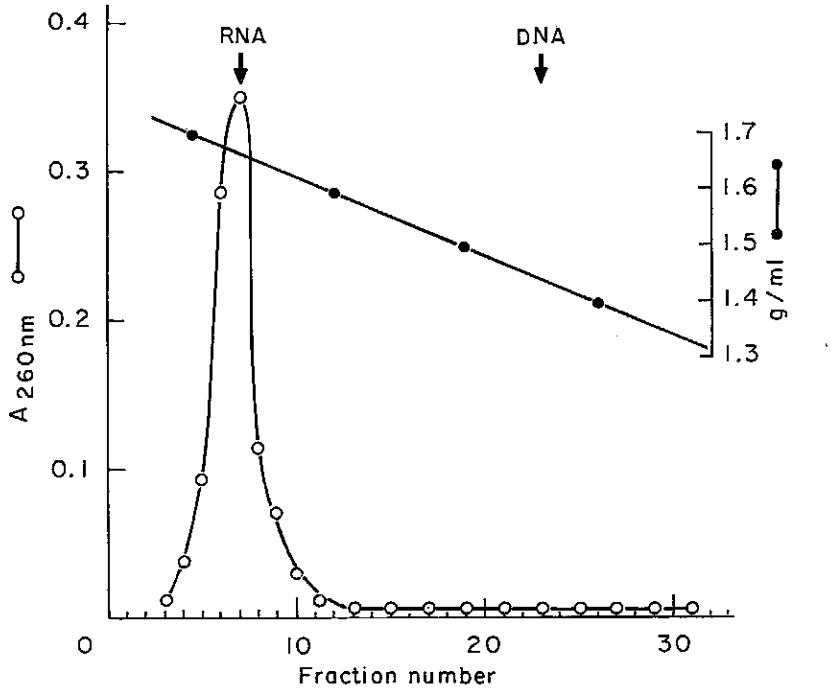

Fig. 4. Cesium sulfate centrifugation of RNA separated from RNA-bound reverse transcriptase. RNA (36 μg) in tris buffer was mixed with Cs_2SO_4 (1.8 g, *p*H 7.3, final volume 3.1 ml) and centrifuged at 20 C (30,000 rpm) for 64 hr in a Spinco SW_{39} rotor. Fractions were collected and analyzed for refractive index and absorbance at 260 nm.

Table VI. Content of DNA in RNA Isolated from RNA-Bound Reverse Transcriptase[a]

DEAE-cellulose peak I	280/260 nm ratio	Amount of RNA (μg) By UV absorption at 260 nm	By orcinol reaction	Diphenylamine reaction[b]
RNA-bound reverse transcriptase	1.40	—	Positive	Negative
RNA separated from the proteins	2.15	410	510	Negative

[a] RNA was separated from the enzyme by phenol, then purified with chloroform, precipitated with alcohol, and dialyzed against distilled water containing 0.2 M KCl. The amount of ribose was determined by the orcinol reaction. The diphenylamine reaction was used to determine whether DNA was present in the RNA preparation.

[b] For the diphenylamine reaction, 1 mg of RNA isolated from RNA-bound reverse transcriptase was used (Burton, 1956).

in excess compared to that found by UV. This is explained by the excess presence of GMP and AMP in the RNA, because it is known that only ribose from these two nucleotides reacts with orcinol (Beljanski, 1949). The RNA bound to reverse transcriptase is as rich in purine nucleotides as transforming RNA itself from *E. coli* (Beljanski and Manigault, 1972; Beljanski *et al.*, 1971*a,b*) (Table VII).

Size of the RNA Bound to Reverse Transcriptase. After centrifugation on a sucrose gradient (Fig. 5), the profile of RNA separated from RNA-bound reverse transcriptase suggests that the UV peak is made by a population of RNAs having similar molecular weights with an average value around 6S, a value which corresponds to that found for transforming RNA excreted by showdomycin-resistant *E. coli* (Beljanski and Manigault, 1972; Beljanski *et al.*, 1971*c*).

Synthesis of DNA on RNA Isolated from RNA-Bound Reverse Transcriptase

Physical Properties of the H^3-Product. After RNA-bound reverse transcriptase was incubated (see Materials and Methods section under Enzyme Assay) in the presence of all four dNTPs, the H^3-product was acid precipitable and resistant to RNase A, Pronase, and 0.3 N KOH at 80 C for 20 min. However, it was degraded by DNase.

Base Ratio of the H^3-DNA-like Product. Base ratio analysis of the H^3-DNA product synthesized on RNA isolated from RNA-bound reverse transcriptase was determined first by the amount of each H^3-dNTP incorporated into TCA-precipitable material (equivalent amounts of each H^3-dNTP were used). Alternatively, the H^3-DNA product was separated from the enzyme by chloroform, extensively dialyzed, treated with 0.3 N KOH to eliminate the RNA, and then analyzed after paper chromatography as previously described (Beljanski and Plawecki, 1973). The base compositions of the RNA template and the DNA product are complementary (Table VII).

H^3-DNA–RNA Hybrid Synthesized During Enzymatic Reaction. After incubation of the RNA-bound reverse transcriptase in the presence of the four H^3-dNTPs under optimal conditions, the enzyme was eliminated by treatment of the incubation mixture with chloroform. The resulting extract containing template RNA and H^3-product was dialyzed and then submitted to equilibrium sedimentation in a Cs_2SO_4 density gradient. TCA-preciptable radioactivity of each fraction was determined, and the peak of radioactive material was found at a density between that of RNA and DNA. After treatment of the H^3-product with alkali, all radioactivity sedimented at the density of DNA (Fig. 6). These observations show that during enzymatic reaction the H^3-DNA is associated with template RNA as a H^3-DNA–RNA hybrid.

20 min). Sucrose gradient analysis (at *p*H 7.65) shows (Fig. 5) that H^3-DNA treated with alkali sediments at around 6S, a value which corresponds to that found for RNA bound to reverse transcriptase.

CONCLUSIONS AND DISCUSSION

We have described some essential characteristics of an RNA-bound reverse transcriptase isolated from *E. coli* Hfr strain. As already established for DNA polymerases I, II, and III, RNA-bound reverse transcriptase requires Mg^{2+} ions and the presence of all four deoxyribonucleoside triphosphates, and its maximal activity is exhibited at *p*H 7.65. The activity strongly decreases in the presence of RNase. The H^3-product synthesized under optimal conditions is TCA precipitable and has characteristics of DNA: sedimentation properties in Cs_2SO_4 alkaline density gradient, sensitivity to DNase, and resistance to RNase and alkali.

On what basis can we exclude the participation of DNA polymerase I, II, or III in the reaction catalyzed by RNA-bound reverse transcriptase?

First, RNA-bound reverse transcriptase is eluted from a DEAE-cellulose column at 0.07 M KCl while DNA polymerase I is eluted at 0.2 M KCl. After passage through G200 Sephadex, RNA-bound reverse transcriptase has an absorbance ratio of 0.7, which corresponds to about 10% nucleic acid content. Activity is RNase sensitive, and no exogenous RNA or DNA templates are required. When RNA-bound reverse transcriptase is resolved into RNA and protein fractions, the activity can be restored by addition to the incubation mixture of either the removed RNA or transforming RNA. Ribosomal RNA and transfer RNA are inactive. RNA which is bound to reverse transcriptase does not contain DNA in a detectable amount, has a high content of A and G nucleotides, and sediments on sucrose density gradients at around 6S.

Second, RNA template separated from the protein fraction and then pretreated with periodate has completely lost its template activity when reincubated with protein fraction. It should be recalled that periodate has no effect at all on DNA itself used as template by DNA polymerases.

Third, the activity of DNA polymerase III is completely abolished by *N*-ethylmaleimide (10 mM) (Gefter *et al.*, 1971), that of DNA polymerase II is decreased to 90% (Kornberg and Gefter, 1971), while that of DNA polymerase I is unaffected. The polymerizing activity of RNA-bound reverse transcriptase was tested in the presence of different compounds, and *N*-ethylmaleimide was found to be without effect. KCl at 0.2 M inhibited the activity only to about 50%, while this salt was without effect on DNA polymerase I and virtually abolished the activity of DNA polymerase II. Rifampicin, rifamycin, and derivatives inhibited to 50–60% the activity of

Beljanski, M. (1972). Synthese *in vitro* de l'ADN sur une matrice d'ARN par une transcriptase d'*E coli*. *Compt. Rend. Acad. Sci. Paris* (Ser. D) **274**:2801.

Beljanski, M., and Manigault, P. (1972). Genetic transformation of bacteria by RNA and loss of oncogenic power properties of *Agrobacterium tumefaciens*: Transforming RNA as template for DNA synthesis. In Beers, R. F. Jr., and Tilghman, R. C. (eds.), *Cellular Modification and Genetic Transformation by Exogenous Nucleic Acids*, Johns Hopkins University Press, Baltimore, p. 81.

Beljanski, M., and Plawecki, M. (1973). Transforming RNA as template directing RNA and DNA synthesis in bacteria. In Niu, M.C., and Segal, S. J., *The Role of RNA in Reproduction and Development*, North Holland Publishing Co., p.203.

Beljanski, M., Beljanski, M., and Bourgarel, P. (1971*a*). Episome à ARN porté par l'ADN d'*Escherichia coli* sauvage et showdomycinoresistant. *Compt. Rend. Acad. Sci. Paris* (*Ser. D*) **272**:2736.

Beljanski, M., Bourgarel, P., and Beljanski, M. (1971*b*). Drastic alteration of ribosomal proteins in showdomycin-resistant *Escherichia coli*. *Proc. Natl. Acad. Sci.* **68**:491.

Beljanski, M., Beljanski, M., and Bourgarel, P. (1971*c*). ARN transformants porteurs de caractères héréditaires chez *E. coli* showdomycino-resistant. *Compt. Rend. Acad. Sci. Paris* (*Ser. D*) **272**:2167.

Beljanski, M., Beljanski, M., Manigault, P., and Bourgarel, P. (1972). Transformation of *Agrobacterium tumefaciens* into a nononcogenic species by an *Escherichia coli* RNA. *Proc. Natl. Acad. Sci.* **69**:191.

Burton, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation and deoxyribonucleic acid. *Biochem. J.* **62**:315.

Cavalieri, L. B., and Caroll, E. (1968). DNA polymerase: Evidence for multiple molecular species. *Proc. Natl. Acad. Sci.* **59**:951.

Cavalieri, L. B., and Caroll, E. (1971). RNA-dependent DNA polymerase from *E. coli* and effect produced by traces of added DNA. *Nature* (*Lond.*) **232**:254.

Duesberg, P., Helm, L. V. D., and Canaani, E. (1971). Comparative properties of RNA and DNA templates for the DNA polymerase of Rous sarcoma virus. *Proc. Natl. Acad. Sci.* **68**:747.

Evans, A. H. (1964). Introduction of specific drug resistance properties by purified RNA containing fractions from pneumococcus. *Proc. Natl. Acad. Sci.* **52**:1442.

Faras, A. J., Taylor, J. M., McDonnell, J. P., Levinsons, W. E., and Bishop, J. M. (1972). Purification and characterization of the deoxyribonucleic acid polymerase associated with Rous sarcoma virus. *Biochemistry* **11**:2334.

Fridlander, B., Fry, M., Bolden, A., and Weissbach, A. (1972). A new synthetic RNA-dependent DNA polymerase from human tissue culture cells. *Proc. Natl. Acad. Sci.* **69**:452.

Gallo, R. (1971). Reverse transcriptase, the DNA polymerase of oncogenic RNA viruses. *Nature* (*Lond.*) **234**:194.

Gallo, R. C., Sarin, P. S., Mangalasseril, M. G., Smith, R. G., Bobrow, S. N., and Reitz, M. S. (1972). Biochemical properties of reverse transcriptase activities from human cells and RNA tumor viruses. In Beers, R. F., Jr., and Tilghman, R. C. (eds.), *Cellular Modification and Genetic Transformation by Exogenous Nucleic Acids*, Johns Hopkins University Press, Baltimore, p. 180.

Gefter, M. L., Hirota, Y., Kornberg, T., Wechsler, J. A., and Barnoux, C. (1971). Analysis of DNA polymerase II and III in mutants of *Escherichia coli* thermosensitive for DNA synthesis. *Proc. Natl. Acad. Sci.* **68**:3150.

Grandgenett, D. P., Gerard, G. F., and Green, M. (1972). A single subunit from avian myeloblastosis virus with both RNA-directed DNA polymerase and ribonuclease H activity. *Proc. Natl. Acad. Sci.* **70**:230.

Gulati, S. C., Axel, R., and Spiegelman, S. (1972). Detection of RNA-instructed DNA polymerase and high molecular weight in malignant tissue. *Proc. Natl. Acad. Sci.* **69**: 2020.

Hurwitz, J., and Leis, J. P. (1972). RNA-dependent DNA polymerase activity of RNA tumor viruses. *J. Virol.* **9**:116.