Viral RNA-dependent DNA Polymerase

Two independent groups of investigators have found evidence of an enzyme in virions of RNA tumour viruses which synthesizes DNA from an RNA template. This discovery, if upheld, will have important implications not only for carcinogenesis by RNA viruses but also for the general understanding of genetic transcription: apparently the classical process of information transfer from DNA to RNA can be inverted.

RNA-dependent DNA Polymerase in Virions of RNA Tumour Viruses

DNA seems to have a critical role in the multiplication and transforming ability of RNA tumour viruses[1]. Infection and transformation by these viruses can be prevented by inhibitors of DNA synthesis added during the first 8–12 h after exposure of cells to the virus[1–4]. The necessary DNA synthesis seems to involve the production of DNA which is genetically specific for the infecting virus[5,6], although hybridization studies intended to demonstrate virus-specific DNA have been inconclusive[1]. Also, the formation of virions by the RNA tumour viruses is sensitive to actinomycin D and therefore seems to involve DNA-dependent RNA synthesis[1–4,7]. One model which explains these data postulates the transfer of the information of the infecting RNA to a DNA copy which then serves as template for the synthesis of viral RNA[1,2,7]. This model requires a unique enzyme, an RNA-dependent DNA polymerase.

No enzyme which synthesizes DNA from an RNA template has been found in any type of cell. Unless such an enzyme exists in uninfected cells, the RNA tumour viruses must either induce its synthesis soon after infection or carry the enzyme into the cell as part of the virion. Precedents exist for the occurrence of nucleotide polymerases in the virions of animal viruses. Vaccinia[8,9]—a DNA virus, Reo[10,11]—a double-stranded RNA virus, and vesicular stomatitis virus (VSV)[12]—a single-stranded RNA virus, have all been shown to contain RNA polymerases. This study demonstrates that an RNA-dependent DNA polymerase is present in the virions of two RNA tumour viruses: Rauscher mouse leukaemia virus (R-MLV) and Rous sarcoma virus. Temin[13] has also identified this activity in Rous sarcoma virus.

Incorporation of Radioactivity from ^{3}H-TTP by R-MLV

A preparation of purified R-MLV was incubated in conditions of DNA polymerase assay. The preparation incorporated radioactivity from ^{3}H-TTP into an acid-insoluble product (Table 1). The reaction required Mg^{2+}, although Mn^{2+} could partially substitute and each of the four deoxyribonucleoside triphosphates was necessary for activity. The reaction was stimulated strongly by dithiothreitol and weakly by NaCl (Table 1). The kinetics of incorporation of radioactivity from ^{3}H-TTP by R-MLV are shown in Fig. 1, curve 1. The reaction rate accelerates for about 1 h and then declines. This time-course may indicate the occurrence of a slow activation of the polymerase in the reaction mixture. The activity is approximately proportional to the amount of added virus.

For other viruses which have nucleotide polymerases in their virions, there is little or no activity demonstrable unless the virions are activated by heat, proteolytic enzymes or detergents[8–12]. None of these treatments increased the activity of the R-MLV DNA polymerase. In fact, incubation at 50° C for 10 min totally inactivated the R-MLV enzyme as did inclusion of trypsin (50 μg/ml.) in the reaction mixture. Addition of as little as 0·01 per cent 'Triton N-101' (a non-ionic detergent) also markedly depressed activity.

Table 1. PROPERTIES OF THE RAUSCHER MOUSE LEUKAEMIA VIRUS DNA POLYMERASE

Reaction system	pmoles ^{3}H-TMP incorporated in 45 min
Complete	3·31
Without magnesium acetate	0·04
Without magnesium acetate + 6 mM $MnCl_2$	1·59
Without dithiothreitol	0·38
Without NaCl	2·18
Without dATP	$<$ 0·10
Without dCTP	0·12
Without dGTP	$<$ 0·10

A preparation of R-MLV was provided by the Viral Resources Program of the National Cancer Institute. The virus had been purified from the plasma of infected Swiss mice by differential centrifugation. The preparation had a titre of $10^{4 \cdot 88}$ spleen enlarging doses (50 per cent end point) per ml. Before use the preparation was centrifuged at 105,000*g* for 30 min and the pellet was suspended in 0·137 M NaCl–0·003 M KCl–0·01 M phosphate buffer (*p*H 7·4)–0·6 mM EDTA (PBS–EDTA) at 1/20 of the initial volume. The concentrated virus suspension contained 3·1 mg/ml. of protein. The assay mixture contained, in 0·1 ml., 5 μmoles Tris-HCl (*p*H 8·3) at 37° C, 0·6 μmole magnesium acetate, 6 μmoles NaCl, 2 μmoles dithiothreitol, 0·08 μmole each of dATP, dCTP and dGTP, 0·001 μmole [^{3}H-*methyl*]–TTP (708 c.p.m. per pmole) (New England Nuclear) and 15 μg viral protein. The reaction mixture was incubated for 45 min at 37° C. The acid-insoluble radioactivity in the sample was then determined by addition of sodium pyrophosphate, carrier yeast RNA and trichloroacetic acid followed by filtration through a membrane filter and counting in a scintillation spectrometer, all as previously described[12]. The radioactivity of an unincubated sample was subtracted from each value (less than 7 per cent of the incorporation in the complete reaction mixture).

Characterization of the Product

The nature of the reaction product was investigated by determining its sensitivity to various treatments. The product could be rendered acid-soluble by either pancreatic deoxyribonuclease or micrococcal nuclease but was unaffected by pancreatic ribonuclease or by alkaline hydrolysis (Table 2). The product therefore has the properties of DNA. If 50 μg/ml. of deoxyribonuclease was

added to a reaction mixture there was no loss of acid-insoluble product. The product is therefore protected from the enzyme, probably by the envelope of the virion, although merely diluting the reaction mixture into 10 mM $MgCl_2$ enables the product to be digested by deoxyribonuclease (Table 2).

Table 2. CHARACTERIZATION OF THE POLYMERASE PRODUCT

Expt.	Treatment	Acid-insoluble radioactivity	Percentage undigested product
1	Untreated	1,425	(100)
	20 μg deoxyribonuclease	125	9
	20 μg micrococcal nuclease	69	5
	20 μg ribonuclease	1,361	96
2	Untreated	1,644	(100)
	NaOH hydrolysed	1,684	100

For experiment 1, 93 μg of viral protein was incubated for 2 h in a reaction mixture twice the size of that described in Table 1, with ^{3}H-TTP having a specific activity of 1,133 c.p.m. per pmole. A 50 μl. portion of the reaction mixture was diluted to 5 ml. with 10 mM $MgCl_2$ and 0·5 ml. aliquots were incubated for 1·5 h at 37° C with the indicated enzymes. (The sample with micrococcal nuclease also contained 5 mM $CaCl_2$.) The samples were then chilled, precipitated with trichloroacetic acid and radioactivity was counted. For experiment 2, two standard reaction mixtures were incubated for 45 min at 37° C, then to one sample was added 0·1 ml. of 1 M NaOH and it was boiled for 5 min. It was then chilled and both samples were precipitated with trichloroacetic acid and counted. In a separate experiment (unpublished) it was shown that the alkaline hydrolysis conditions would completely degrade the RNA product of the VSV virion polymerase.

Localization of the Enzyme and its Template

To investigate whether the DNA polymerase and its template were associated with the virions, a R-MLV suspension was centrifuged to equilibrium in a 15–50 per cent sucrose gradient and fractions of the gradient were assayed for DNA polymerase activity. Most of the activity was found at the position of the visible band of virions (Fig. 2). The density at this band was 1·16 g/cm^3, in agreement with the known density of the virions[14]. The polymerase and its template therefore seem to be constituents of the virion.

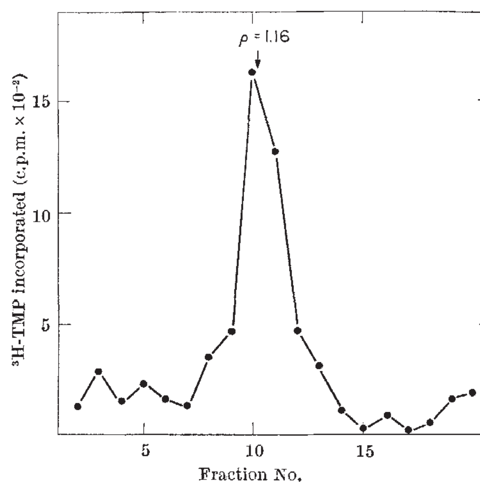

Fig. 2. Localization of DNA polymerase activity in R-MLV by isopycnic centrifugation. A preparation of R-MLV containing 150 μg of protein in 50 μl. was layered over a linear 5·2 ml. gradient of 15-50 per cent sucrose in PBS–EDTA. After centrifugation for 2 h at 60,000 r.p.m. in the Spinco 'SW65' rotor, 0·27 ml. fractions of the gradient were collected and 0·1 ml. portions of each fraction were incubated for 60 min in a standard reaction mixture. The acid-precipitable radioactivity was then collected and counted. The density of each fraction was determined from its refractive index. The arrow indicates the position of a sharp, visible band of light-scattering material which occurred at a density of 1·16.

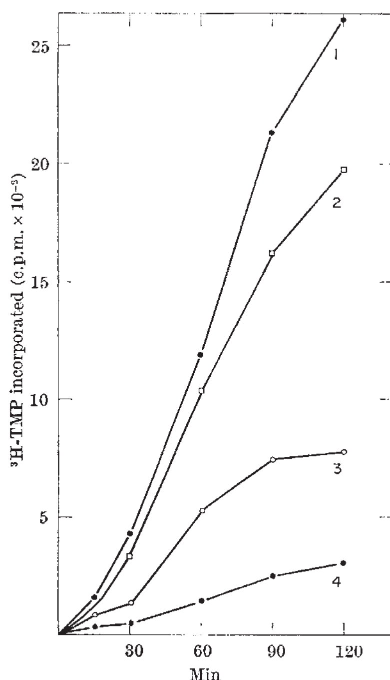

Fig. 1. Incorporation of radioactivity from ^{3}H-TTP by the R-MLV DNA polymerase in the presence and absence of ribonuclease. A 1·5-fold standard reaction mixture was prepared with 30 μg of viral protein and ^{3}H-TTP (specific activity 950 c.p.m. per pmole). At various times, 20 μl. aliquots were added to 0·5 ml. of non-radioactive 0·1 M sodium pyrophosphate and acid insoluble radioactivity was determined[12]. For the preincubated samples, 0·06 ml. of H_2O and 0·01 ml. of R-MLV (30 μg of protein) were incubated with or without 10 μg of pancreatic ribonuclease at 22° C for 20 min, chilled and brought to 0·15 ml. with a concentrated mixture of the components of the assay system. Curve 1, no treatment; curve 2, preincubated; curve 3, 10 μg ribonuclease added to the reaction mixture; curve 4, preincubated with 10 μg ribonuclease.

The Template is RNA

Virions of the RNA tumour viruses contain RNA but no DNA[15,16]. The template for the virion DNA polymerase is therefore probably the viral RNA. To substantiate further that RNA is the template, the effect of ribonuclease on the reaction was investigated. When 50 μg/ml. of pancreatic ribonuclease was included in the reaction mixture, there was a 50 per cent inhibition of activity during the first hour and more than 80 per cent inhibition during the second hour of incubation (Fig. 1, curve 3). If the virions were preincubated with the enzyme in water at 22° C and the components of the reaction mixture were then added, an earlier and more extensive inhibition was evident (Fig. 1, curve 4). Preincubation in water without ribonuclease caused only a slight inactivation of the virion polymerase activity (Fig. 1, curve 2). Increasing the concentration of ribonuclease during preincubation could inhibit more than 95 per cent of the DNA polymerase activity (Table 3). To ensure that the inhibition by ribonuclease was attributable to the enzymic activity of the added protein, two other basic proteins were preincubated with the virions. Only ribonuclease was able to inhibit the reaction (Table 3). These experiments substantiate the idea that RNA is the template for the reaction. Hybridization experiments are in progress to determine if the DNA is complementary in base sequence to the viral RNA.

Ability of the Enzyme to Incorporate Ribonucleotides

The deoxyribonucleotide incorporation measured in these experiments could be the result of an RNA polymerase activity in the virion which can polymerize deoxyribonucleotides when they are provided in the reaction mixture. The VSV RNA polymerase and the R-MLV DNA polymerase were therefore compared. The VSV RNA polymerase incorporated only ribonucleotides. At its pH optimum of 7·3 (my unpublished observation),

in the presence of the four common ribonucleoside triphosphates, the enzyme incorporated ^{3}H-GMP extensively[12]. At this pH, however, in the presence of the four deoxyribonucleoside triphosphates, no ^{3}H-TMP incorporation was demonstrable (Table 4). Furthermore, replacement of even a single ribonucleotide by its homologous deoxyribonucleotide led to no detectable synthesis (my unpublished observation). At pH 8·3, the optimum for the R-MLV DNA polymerase, the VSV polymerase catalysed much less ribonucleotide incorporation and no significant deoxyribonucleotide incorporation could be detected.

Table 3. EFFECT OF RIBONUCLEASE ON THE DNA POLYMERASE ACTIVITY OF RAUSCHER MOUSE LEUKAEMIA VIRUS

Conditions	pmoles ^{3}H-TMP incorporation
No preincubation	2·50
Preincubated with no addition	2·20
Preincubated with 20 μg/ml. ribonuclease	0·69
Preincubated with 50 μg/ml. ribonuclease	0·31
Preincubated with 200 μg/ml. ribonuclease	0·08
Preincubated with no addition	3·69
Preincubated with 50 μg/ml. ribonuclease	0·52
Preincubated with 50 μg/ml. lysozyme	3·67
Preincubated with 50 μg/ml. cytochrome c	3·97

In experiment 1, for the preincubation, 15 μg of viral protein in 5 μl. of solution was added to 45 μl. of water at 4° C containing the indicated amounts of enzyme. After incubation for 30 min at 22° C, the samples were chilled and 50 μl. of a 2-fold concentrated standard reaction mixture was added. The samples were then incubated at 37° C for 45 min and acid-insoluble radioactivity was measured. In experiment 2, the same procedure was followed, except that the preincubation was for 20 min at 22° C and the 37° C incubation was for 60 min.

Table 4. COMPARISON OF NUCLEOTIDE INCORPORATION BY VESICULAR STOMATITIS VIRUS AND RAUSCHER MOUSE LEUKAEMIA VIRUS

Precursor	pH	Incorporation in 45 min (pmoles) Vesicular stomatitis virus	Mouse leukaemia virus
^{3}H-TTP	8·3	< 0·01	2·3
^{3}H-TTP (omit dATP)	8·3	N.D.	0·06
^{3}H-TTP (omit dATP; plus ATP)	8·3	N.D.	0·08
^{3}H-GTP	8·3	0·43	< 0·03
^{3}H-GTP	7·3	3·7	< 0·03

When ^{3}H-TTP was the precursor, standard reaction conditions were used (see Table 1). When ^{3}H-GTP was the precursor, the reaction mixture contained, in 0·1 ml., 5 μmoles Tris-HCl (pH as indicated), 0·6 μmoles magnesium acetate, 0·3 μmoles mercaptoethanol, 9 μmoles NaCl, 0·08 μmole each of ATP, CTP, UTP; and 0·001 μmole ^{3}H-GTP (1,040 c.p.m. per pmole). All VSV assays included 0·1 per cent 'Triton N-101' (ref. 12) and 2–5 μg of viral protein. The R-MLV assays contained 15 μg of viral protein.

The R-MLV polymerase incorporated only deoxyribonucleotides. At pH 8·3, ^{3}H-TMP incorporation was readily demonstrable but replacement of dATP by ATP completely prevented synthesis (Table 4). Furthermore, no significant incorporation of ^{3}H-GMP could be found in the presence of the four ribonucleotides. At pH 7·3, the R-MLV polymerase was also inactive with ribonucleotides. The polymerase in the R-MLV virions is therefore highly specific for deoxyribonucleotides.

DNA Polymerase in Rous Sarcoma Virus

A preparation of the Prague strain of Rous sarcoma virus was assayed for DNA polymerase activity (Table 5). Incorporation of radioactivity from ^{3}H-TTP was demonstrable and the activity was severely reduced by omission of either Mg^{2+} or dATP from the reaction mixture. RNA-dependent DNA polymerase is therefore probably a constituent of all RNA tumour viruses.

These experiments indicate that the virions of Rauscher mouse leukaemia virus and Rous sarcoma virus contain a DNA polymerase. The inhibition of its activity by ribonuclease suggests that the enzyme is an RNA-dependent DNA polymerase. It seems probable that all RNA tumour viruses have such an activity. The existence of this enzyme strongly supports the earlier suggestions[1-7] that genetically specific DNA synthesis is an early event in the replication cycle of the RNA tumour viruses and that DNA is the template for viral RNA synthesis. Whether the viral DNA ("provirus")[2] is integrated into the host genome or remains as a free template for RNA synthesis will require further study. It will also be necessary to determine whether the host DNA-dependent RNA polymerase or a virus-specific enzyme catalyses the synthesis of viral RNA from the DNA.

Table 5. PROPERTIES OF THE ROUS SARCOMA VIRUS DNA POLYMERASE

Reaction system	pmoles ^{3}H-TMP incorporated in 120 min
Complete	2·06
Without magnesium acetate	0·12
Without dATP	0·19

A preparation of the Prague strain (sub-group C) of Rous sarcoma virus[16] having a titre of 5 × 10^7 focus forming units per ml. was provided by Dr Peter Vogt. The virus was purified from tissue culture fluid by differential centrifugation. Before use the preparation was centrifuged and the pellet dissolved in 1/10 of the initial volume as described for the R-MLV preparation. For each assay 15 μl. of the concentrated Rous sarcoma virus preparation was assayed in a standard reaction mixture by incubation for 2 h. An unincubated control sample had radioactivity corresponding to 0·14 pmole which was subtracted from the experimental values.

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RNA-dependent DNA Polymerase in Virions of Rous Sarcoma Virus

INFECTION of sensitive cells by RNA sarcoma viruses requires the synthesis of new DNA different from that synthesized in the S-phase of the cell cycle (refs. 1, 2 and unpublished results of D. Boettiger and H. M. T.); production of RNA tumour viruses is sensitive to actinomycin D[3,4]; and cells transformed by RNA tumour viruses have new DNA which hybridizes with viral RNA[5,6]. These are the basic observations essential to the DNA provirus hypothesis—replication of RNA tumour viruses takes place through a DNA intermediate, not