DNA (unpublished results of J. R.). Thus the origin of these molecules as well as the DNA-dependent DNA polymerase remains an open question.

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Synthetic DNA–RNA Hybrids and RNA–RNA Duplexes as Templates for the Polymerases of the Oncogenic RNA Viruses

by

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Six RNA viruses have now been shown to contain DNA polymerase activities directed by single-stranded RNA, double-stranded RNA and double-stranded DNA. It is further demonstrated that DNA–RNA hybrids, such as synthetic dC·rG, act as even more effective templates.

The recent reports by Temin[1] and Baltimore[2] of an RNA-directed DNA polymerase in oncogenic RNA viruses have been confirmed and extended[3]. We established that the DNA synthesized is complementary to the viral RNA and that DNA–RNA hybrids appear as early intermediates of the reaction. Both features argue compellingly that viral RNA functions as a template in the synthesis of the DNA.

The RNA–DNA hybrids detected as presumptive intermediates are not likely to be integrated as such into the genome, an event necessary to explain the heritably stable state of the transformation induced by these oncogenic viruses. The required conversion of the RNA–DNA hybrid into a DNA–DNA duplex demands a DNA-directed DNA polymerase. We already had[3] suggestive evidence that DNA strands synthesized late in the reaction were identical rather than complementary to the viral RNA. We set out to find the DNA-directed DNA polymerase implied by these observations and detected[4] the relevant activity in all six oncogenic viruses examined. The DNA-directed polymerase activity thus revealed had a number of interesting properties. There was a clear preference for double-stranded DNA and, except for covalently linked circles, virtually any double-stranded DNA was accepted as a template. In each case, it could be shown by appropriate hybridizations that the DNA synthesized was complementary to the DNA used as the initiating template.

We were still left with the problem of identifying the polymerization reaction which converts the DNA–RNA hybrids into the DNA–DNA duplex. To synthesize natural DNA–RNA hybrids in amounts adequate for enzymatic test is still a formidable task. To avoid the logistic problems involved we decided to use synthetic polynucleotide duplexes. There is ample evidence[5–7] of their usefulness in studies of DNA and RNA synthesis, and the DNA polymerase of *E. coli* has been shown to use the synthetic ribopolymer rA·rU to synthesize the deoxyribopolymer dA·dT (ref. 8). We have found that certain of the synthetic duplexes are not only functional, but are superior to natural templates by almost two orders of magnitude in stimulating polymerization. This finding leads to an extraordinarily useful tool for detecting the enzyme activity. We summarize here the results of our investigations.

Comparison of Templates

The viruses used in the preparation of enzymes were avian myeloblastosis virus (AMV), feline leukaemia virus (FeLV), Moloney sarcoma virus (MSV), Rauscher leukaemia virus (RLV), mouse mammary tumour virus (MTV) and a rat mammary tumour virus (RMTV). Table 1 illustrates the responses of the six RNA oncogenic viral preparations to the resident RNA templates (column 1), exogenous DNA (column 2), and to the hybrid duplex (column 3) formed from polydeoxyadenylate and polyriboguanylate (dC·rG).

The stimulation observed with CEF–DNA is not maximal because for purposes of comparison it was held at the same level (4 μg/ml.), at which the reaction with the dC·rG is optimal. For all six viruses there is clear evidence of a hybrid directed DNA synthesis that is dramatically

superior to the reactions previously studied. The kinetics of DNA synthesis with the three templates (Fig. 1) emphasize the magnitude of the difference in template capabilities. When plotted on the same scale, the synthetic activities observed with the resident viral RNA and CEF–DNA are almost trivial compared with dC·rG.

We examined the template capabilities of as many synthetic polynucleotides as possible. Table 2 shows the response to single homopolymers. It should be noted that in this, as in the tables to be discussed, all measurements involved examinations over 20–30 min periods with samples taken every 5–7 min. The 5 min values are recorded to permit better comparison with those cases in which the kinetics diverge from linearity after 10 min. In all cases shown in this and subsequent tables, both complementary deoxyriboside triphosphates were included in the assay, one being identified by an α-^{32}P label, and the other by a ^{3}H label.

Table 2 demonstrates that of the ribohomopolymers, only rC possesses detectable template activity and here the synthesis lasts only for 5 min. In the case of the deoxyribopolymers, poly dC supports extensive and prolonged synthesis of poly dG, and some later incorporation of dC. Although much smaller, both poly dA and poly dI stimulate the incorporation of the corresponding complementary nucleotides.

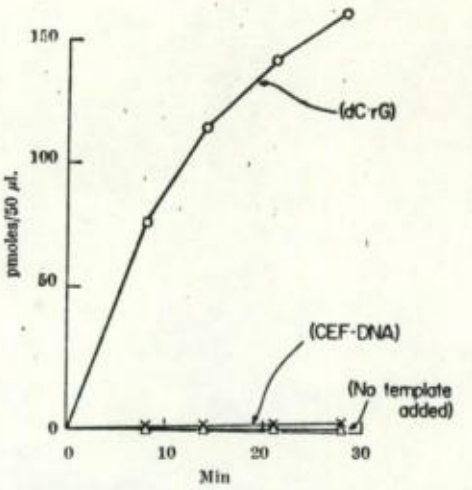

Fig. 1. Kinetics of incorporation of α-^{32}P-dGTP by RLV polymerase. A 0·25 ml. standard reaction mixture was used. The specific radioactivity of α-^{32}P-dGTP was 60 c.p.m./pmole. Methods of preparation of CEF-DNA and dC · rG are described in the legend to Table 1. At the indicated times, 50 µl. samples were withdrawn and the acid precipitable radioactivity was determined as described[3].

The abilities of various synthetic double-stranded DNA and RNA homopolymers to serve as templates are recorded in Table 3. Except for rC·rG, all stimulate some incorporation of one of the complementary nucleotide pairs. Of those tested, rI·rC is clearly the best, followed by dC·dG and dC·dI. It is interesting to note that, with the exception of rC·rG, the polyribopolymers are superior to the corresponding deoxypolymers as templates. Table 4 compares the template capabilities of a variety of synthetic DNA–RNA hybrid duplexes. Some of these are the best templates, notably dC·rG and dI·rC.

Table 1. RESPONSES OF ONCOGENIC VIRAL RNA POLYMERASES TO ADDED DNA AND A SYNTHETIC RNA–DNA HYBRID

Polymerase	pmoles nucleotide incorporated in 10 min/10 µg of viral protein		
	Template		
	None	CEF–DNA	dC·rG
AMV	2·8	5·3	140·0
FeLV	2·1	2·6	39·7
MSV	1·2	1·2	30·0
RLV	1·5	4·3	162·0
MTV	0·1	1·0	35·1
R-MTV	0·1	0·6	21·4

A standard incubation mixture of 0·25 ml. contains 12·5 µmoles Tris-HCl (pH 8·3); 3 µmoles $MgCl_2$; 10 µmoles KCl; 2·5 µmoles dithiothreitol; 0·04 µmoles of each of the deoxyribonucleoside triphosphates. To monitor the reaction, α-^{32}P-dGTP was used at a specific activity of 65 c.p.m./pmole. Reactions that were stimulated by added templates were carried out by including the template at a level of 1·0 µg per 0·25 ml. in the standard incubation mixture. The incorporations noted above represent those observed in 50 µl. aliquots corresponding to 10 µg of viral protein. Virus particles suspended in 0·01 M Tris (pH 8·3)—0·1 M NaCl—0·002 M EDTA (TNE) at a concentration of 2·3 mg of viral protein per ml. were preincubated for 10 min at 0° C in the presence of 0·2 per cent Nonidet P-40 and 0·1 M dithiothreitol. The incubated solution was then added to a standard incubation mixture at a level of 50 µg of viral protein per 0·25 ml. and incubated at 37° C. After incubation for 10 min, 50 µl. aliquots were withdrawn and processed for determining the acid precipitable radioactivity as described elsewhere[3]. The particles designated here as R-MTV were obtained from supernatant fluids of rat mammary tumour[4], R-35 grown *in vitro* at Charles Pfizer Co., Maywood, New Jersey. The source of the other viruses and their methods of purification have been described previously[3]; R-MTV was purified in the same way as the monkey mammary tumour virus. dC·rG was made by mixing equal amounts of poly dC and poly rG (100 µg per ml. in 0·01 M Tris, pH 7·4), making the solution 0·2 M with respect to NaCl and allowing the mixture to stand for 15 min at room temperature. The CEF DNA used was made from trypsinized cells from chick embryos according to the procedure of Gillespie and S.S.[10].

Table 2. RESPONSE OF AMV-POLYMERASE TO SINGLE-STRANDED HOMOPOLYMERS

Template	Incorporation (pmoles/5 min/10 µg viral protein)			
rA	dT	(<0·1)	dA	(<0·1)
rC	dG	(3·8)	dC	(<0·1)
rG	dC	(<0·1)	dG	(<0·1)
rI	dC	(<0·1)	dG	(<0·1)
rU	dA	(<0·1)	dT	(<0·1)
dA	dT	(1·2)	dA	(0·2)
dC	dG	(11·0)	dC	(0·8)
dG	dC	(<0·1)	dG	(<0·1)
dI	dC	(1·2)	dG	(<0·1)
dT	dA	(<0·1)	dT	(<0·1)

The composition of the reaction mixtures was as described for Table 1, except that in each experiment only the two deoxyriboside triphosphates were used which corresponded to the identity and the complement of the bases in the template. The template concentration was 1 µg/0·25 ml. of the incubation mixture. The labels used were: ^{3}H-dATP (115 c.p.m./pmole), ^{3}H-dCTP (125 c.p.m./pmole), ^{32}P-dGTP (60 c.p.m./pmole), and ^{32}P-dTTP (110 c.p.m./pmole). 50 µl. of the reaction mixture was withdrawn and acid precipitable radioactivity was determined as described[3] after 5 min of incubation at 37° C.

Table 3. RESPONSE OF AMV-POLYMERASE TO SYNTHETIC DOUBLE-STRANDED DNA AND RNA POLYMERS

Template	Incorporation (pmoles/5 min/10 µg viral protein)			
rA · rU	dT	(15·0)	dA	(0·4)
rC · rG	dG	(<0·1)	dC	(<0·1)
rI · rC	dG	(128·0)	dC	(2·1)
dA · dT	dT	(0·8)	dA	(1·4)
dC · dG	dG	(26·9)	dC	(0·4)
dC · dI	dG	(24·9)	dC	(2·0)

The reaction mixtures and conditions of reactions were as described for Table 1. The duplexes were prepared by using the appropriate polymer components, using the method described for dC·rG (see legend to Table 1). The specific activities of the deoxyriboside triphosphates used were the same as those for Table 2, and the appropriate complementary pair of ^{3}H and ^{32}P-labelled nucleoside triphosphates were present in each reaction.

Table 4. RESPONSE OF AMV-POLYMERASE TO SYNTHETIC HYBRIDS

Template	Incorporation (pmoles/5 min/10 µg viral protein)			
dA · rU	dT	(0·8)	dA	(<0·1)
dD · rG	dG	(141·0)	dC	(1·9)
dC · rI	dG	(14·5)	dC	(1·9)
dG · rC	dC	(<0·1)	dG	(<0·1)
dI · rC	dC	(1·3)	dG	(104·6)
dT · rA	dA	(0·9)	dT	(10·3)

The reaction mixtures and conditions of reactions were the same as described in the legend to Table 1. The hybrids were prepared using the method described for making dC · rG in the legend to Table 1. All other details are as described in the legends to Tables 2 and 3.

There is a striking preference for dGTP and rGTP as substrates. This recalls an analogous situation observed with the Qβ replicase[8] and the DNA-dependent RNA polymerase[7], both of which synthesize poly G if provided with poly C as a template. The preference for dG may

Table 5. TEST OF AMV POLYMERASE TO INCORPORATE RIBONUCLEOTIDES WITH SYNTHETIC POLYNUCLEOTIDE TEMPLATES

Template	Incorporation (pmoles/90 min/10 μg viral protein) Ribonucleotides		Deoxyribonucleotides
rA · rU	rU (<0·1)	rA (<0·1)	dT (73·6)
rI · rC	rC (<0·1)	rG (<0·2)	dG (326·0)
dA · dT	rU (<0·1)	rA (<0·1)	dT (9·0)
dC · dG	rG (<0·1)	rC (<0·1)	dG (109·0)
dC · rG	rG (<0·1)	rC (<0·1)	dG (322·0)
dT · rA	rA (<0·1)	rU (<0·1)	dT (49·7)

The reaction mixtures and the conditions of experiments were as for Table 1. Here, however, riboside triphosphates were used together with their deoxy analogues. Reactions in the absence of deoxyriboside triphosphates gave the same results. α-^{32}P-labelled deoxyriboside triphosphates and ^{3}H-labelled riboside triphosphates were used to monitor the polyribonucleotide synthesis, and the specific activities for the rATP, rGTP, rCTP, and rUTP were all at 120 c.p.m./pmole. The α-^{32}P-dGTP and α-^{32}P-dTTP used to follow the DNA synthesis were at 60 c.p.m./pmole.

indicate that guanosine is the 5′ terminus and is therefore the first nucleotide inserted. This is in fact the case with the Qβ replicase.

Asymmetric Copying

Tables 3 and 4 show a marked asymmetry in the use of the substrates provided. In no case are the two complementary nucleoside triphosphates incorporated with equal facility. The asymmetry, however, is not absolute, even in the 5 min results recorded in Tables 3 and 4. We found that although there was an initial preference for the incorporation of ^{32}P-dT, ^{3}H-dA is polymerized. A very similar phenomenon is observed (Fig. 2) with ^{32}P-dG and ^{3}H-dC in polymerizations by dI·rC. In some cases, synthesis prolonged for 90 min or more leads to eventual equalization of the incorporation of the two substrates. It should be noted that the use of the preferred substrate (for example, dTTP by dT·rA or dGTP by dI·rC) is not strikingly influenced by the presence of the complementary nucleoside triphosphate in the reaction mixture. As expected, there is symmetrical use and a requirement for the presence of both complementary substrates when an alternating copolymer is used as a template (Fig. 3). Here both complementary nucleotides are incorporated from the very onset at almost equal rates and molarities. Further, unlike the homopolymeric duplexes, leaving out either one of the complementary substrates leads to the complete loss of synthetic activity.

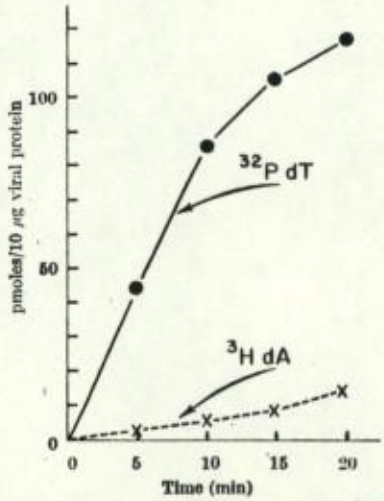

Fig. 2. Kinetics of incorporation of α-^{32}P-TTP and ^{3}H-dATP by AMV polymerase with dT · rA template. A 0·25 ml. standard reaction mixture was used. The specific radioactivities of α-^{32}P-TTP and ^{3}H-dATP were 110 c.p.m./pmole and 115 c.p.m./pmole, respectively. The method of preparation of the hybrid template is described in the legend to Table 1. At the indicated times, 50 μl. samples were withdrawn and the acid precipitable radioactivity was determined as described earlier[2].

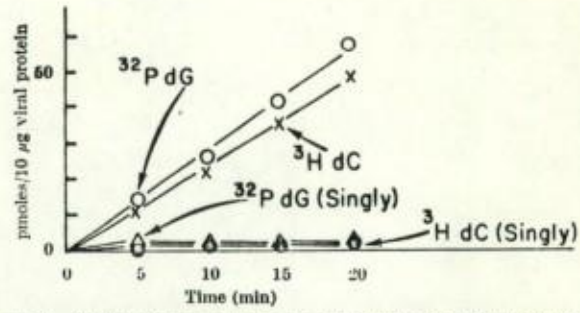

Fig. 3. Kinetics of incorporation of α-^{32}P-dGTP and ^{3}H-dCTP by AMV polymerase with d(I–C) as template. A 0·25 ml. standard reaction mixture was used. The specific radioactivities of α-^{32}P-dGTP and ^{3}H-dCTP were 60 c.p.m./pmole and 110 c.p.m./pmole respectively. At the indicated times, 50 μl. aliquots were withdrawn and the TCA precipitable radioactivity was determined as described earlier[2]. Results are expressed as pmoles of nucleotide incorporated/10 μg viral protein. ○ and × indicate the incorporation obtained when both deoxyribonucleoside triphosphates are present. △ and ● refer to the incorporation obtained when one nucleotide only is present.

Inability to Incorporate Ribonucleotides

It has already been noted[1–3] that the oncogenic polymerases do not synthesize ribopolymers using the viral RNA as a template. In view of the vastly superior template capabilities of some of the synthetic polymers, this question was re-examined and the results are recorded in Table 5. Even after 90 min, no signs of ribonucleotide incorporation could be detected with any of the templates during a period in which excellent polymerization (last column of Table 5) was occurring with the deoxyribonucleotides.

Uses for Synthetic Templates

A detailed examination of the intermediates in the reactions mediated by these synthetic duplexes should be very useful for the study of the chemistry of the polymerization and the factors which determine its asymmetry. Even a casual inspection of Table 1 and Fig. 1 reveals the obvious advantage of using templates such as dC·rG for detecting enzyme in cells or virus particles and following activity during purification procedures. We have now established that oncogenic viruses contain DNA polymerase activities directed by single-stranded RNA, double-stranded RNA, double-stranded DNA, and DNA–RNA hybrids. Further, all oncogenic RNA viruses examined contain all these activities. The number of different kinds of protein molecules required to handle this variety of templates remains to be determined.

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