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DNA-directed DNA Polymerase Activity in Oncogenic RNA Viruses

by

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Besides having RNA-dependent DNA polymerase activity, oncogenic RNA viruses possess a DNA-directed DNA polymerase which is distinguished from previously described enzymes of this type in preferring double-stranded DNA as template and yielding a principally double-stranded product.

Temin[1] and Baltimore[2] recently reported the existence of an RNA-directed DNA polymerase in oncogenic RNA viruses. We confirmed these findings with six oncogenic viruses and further established[3] the following features of the reaction: (*a*) physical and chemical characterizations proved that the product was in fact a DNA heteropolymer; (*b*) molecular hybridization showed that the DNA synthesized was complementary to the viral RNA contained in the enzyme preparation; and (*c*) RNA–DNA complexes were detected as early components in the polymerization. The specific complementarity of the synthetic DNA to viral RNA and the early appearance of RNA–DNA hybrids implied that the viral RNA functioned as a template in the synthesis of the DNA.

The heritably stable state that characterizes cells transformed by these oncogenic agents seems to require integration of the newly synthesized DNA into the genome of the cell. It is unlikely that the RNA–DNA hybrids detected as presumptive intermediates in the reaction can serve this purpose. It is more likely that the single-stranded DNA has to be converted into its double-stranded equivalent for integration, a conversion that would necessitate a DNA-directed DNA polymerase. We therefore pointed[3] to the need to search for such an enzyme, either in the virion or in the infected cell, which would presumably be characterized by its ability to use duplexes to generate duplexes. We have now examined six oncogenic RNA viruses—Rauscher leukaemia virus (RLV), Rous sarcoma virus RSV-(RAV-1), avian myeloblastosis virus (AMV), murine mammary tumour virus (MTV), Moloney sarcoma virus (MSV) and the feline leukaemia virus (FeLV)—and encountered a DNA-directed DNA polymerase in the virions of all of them. We report here some of the properties of the reactions and the products formed.

Previously[3], we alluded to two observations in prolonged syntheses that could not be explained solely by the RNA-directed DNA polymerase activity we were studying. The base composition of the product of an extensive synthesis seemed to be more identical than complementary to the viral RNA. This implied that the DNA complement made early in the reaction was subsequently used as a template for the formation of a complement to itself. In addition, although early products are all hybridizable, late DNA components appear that do not complex to viral RNA (unpublished observations). The implication is that these late DNA products are either duplexes or single strands identical in sequence to the viral RNA. All this suggests the presence of a DNA-directed DNA synthesis in which the DNA formed initially serves as a template for subsequent polymerization.

Evidence for a DNA-directed DNA Polymerase

In the experiment documented in Table 1, the addition of either *Escherichia coli* DNA or mouse embryo fibroblast (MEF) DNA results in a striking stimulation of ^{3}H-dATP incorporation. A trivial explanation of this would invoke protection by the added DNA against nucleolytic degradation of the DNA synthesized by the RNA-directed polymerase. To test—and eliminate—this, and at the same time provide a more convenient system for studying the DNA-directed step, it was necessary to eliminate the RNA-directed activity. This can be done by destroying the resident viral RNA by previous treatment of the disrupted virions with a suitable nuclease. Micrococcal nuclease is convenient because it requires Ca^{2+}. Its activity can consequently be readily neutralized by the specific chelating agent ethyleneglycol-*bis*-(aminoethyl ether)tetraacetic acid (EGTA). Fig. 1 shows the kinetics of DNA synthesis in four oncogenic viral preparations that had been pretreated with micrococcal enzyme. There is little residual activity in the absence of added exogenous nucleic acid; the addition of DNA leads to an excellent response in all four enzyme preparations.

Table 2 shows that the DNA-stimulated reaction requires all four deoxyriboside triphosphates as well as Mg^{2+}. The last control recorded in Table 2, omission of the viral enzyme, was included to eliminate the unlikely possibility that the enzyme we were detecting was present as a contaminant of the micrococcal nuclease used to eradicate the RNA-directed step.

Table 1. STIMULATION OF THE AMV POLYMERASE BY DNA

DNA	c.p.m. −DNA	c.p.m. +DNA
E. coli (4·0 μg)	631	3,460
MEF (1·5 μg)	840	4,340

A standard incubation mixture of 1 ml. contains in μmoles: 50 Tris–HCl (*p*H 8·3), 12 $MgCl_2$, 40 KCl, 2 dithiothreitol, 0·8 each of the non-labelled deoxyribonucleoside triphosphates and 0·04 labelled deoxyribonucleotide. In this experiment, ^{3}H-dATP was used at a specific activity of 330 c.p.m./pmole. The incorporations represent those observed in 0·1 ml. aliquots, corresponding to 15 μg viral protein. Virus particles suspended in 0·01 M Tris (*p*H 8·3) at 300 μg viral protein/ml. were preincubated 10 min at 0° C in the presence of 0·2 per cent 'Nonidet P-40' detergent with dithiothreitol at 30 mM. The virus was then added to a standard incubation mixture at a level of 130 μg/ml. and incubated at 37° C for 30 min. The reaction was terminated by the addition of 0·5 ml. water and 0·3 ml. trichloroacetic acid (TCA) mixture (equal volume mixture of 100 per cent TCA solution, saturated sodium orthophosphate, and saturated sodium pyrophosphate). After 10 min, the precipitable radioactivity was collected on a nitrocellulose filter, dried, and the radioactivity determined in a liquid scintillation counter using BBOT-toluene scintillation fluid. Mouse embryo fibroblast DNA and *E. coli* DNA were extracted following the procedure of Gillespie and S.S. and added to the reaction mixtures as indicated.

Table 2. REQUIREMENTS OF DNA-DIRECTED DNA POLYMERASE

Conditions	^{3}H-dAMP incorporated (c.p.m.)
Complete (12 mM Mg^{2+}, 40 mM K^+)	
1 μg MEF DNA	404
−dCTP	55
−dGTP	15
−dATP	41
−Mg^{2+}	62
−Viral polymerase	30

Enzyme reactions were carried out as described in Table 1. The virus suspension was treated with micrococcal nuclease before incubation at 37° C, as described in Fig. 1. The specific activity of the ^{3}H-dATP was 330 c.p.m./pmole.

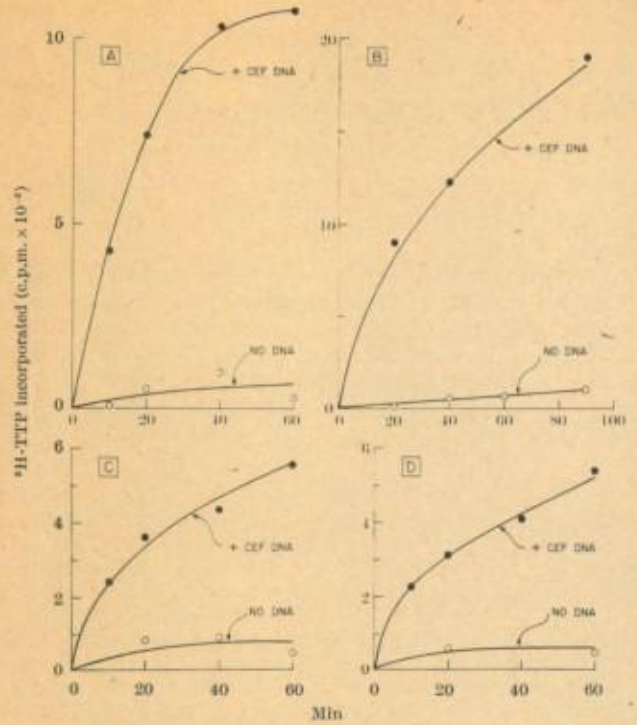

Fig. 1. Kinetics of incorporation of ^{3}H-TTP by DNA-directed DNA polymerases from (*A*) RSV-(RAV-1), (*B*) AMV, (*C*) RLV and (*D*) MTV. For each reaction, a 0·25 ml. of a standard reaction mixture (Table 1) was used. The specific radioactivity of ^{3}H-TTP was $1·4 \times 10^3$ c.p.m./pmole. Chick embryo fibroblast DNA at a concentration of 3 μg/0·25 ml. reaction was used as template for the four DNA–DNA polymerases. The DNA was prepared as described previously[7] from trypsinized chick embryos. The preparation, source, and purification of the viruses used have been described previously. To eliminate the RNA-directed reaction, all the virus preparations used were submitted to a non-ionic detergent treatment ('NP-40', Shell Co.) for 10 min at 0° C in the presence of 0·1 M dithiothreitol in volumes of about 25 μl. The 'NP-40' concentrations were 0·2 per cent for RSV-(RAV-1) and AMV, 0·1 per cent for RLV, and 0·17 per cent for MTV. These were then diluted to 250 μl. for digestion with nuclease from *Staphylococcus aureus* (Worthington); incubation was at room temperature for 30 min in the presence of 240 μg nuclease/ml. and 0·002 M Ca^{2+}. The nuclease action was stopped by adding EGTA to a final concentration of 0·004 M. Nucleoside triphosphates were then added and incorporation kinetics followed at 37° C. The amounts of viral protein per 30 μl.: *A*, 3·75 μg; *B*, 3·90 μg; *C*, 2·56 μg and *D*, 2·80 μg. At the indicated times, 30 μl. aliquots were withdrawn, precipitated with TCA in the presence of 60 μg of *E. coli* RNA as carrier, processed and counted as described in Table 1.

Nature of the Product

The product of an extensive synthesis was purified by phenol extraction and alcohol precipitation. It was then subjected to physical and enzymological tests. Table 3 shows that the acid-precipitable product can be degraded by deoxyribonuclease and spleen phosphodiesterase but not by ribonuclease or alkali. To confirm that it is DNA, the density of the product was examined by equilibrium density centrifugation in Cs_2SO_4 and the result is shown in Fig. 2. *E. coli* DNA was included as a density marker (1·426 g cm^{-3}). The density of the DNA synthesized in response to the addition of MEF DNA is found at the same density (1·420) as MEF DNA. The DNA produced by the RNA-directed reaction has[3] the indicated density of 1·450.

The average size of the product is compared (Fig. 3) with that of template in neutral and alkaline sucrose gradients. It is clear that the radioactive product is smaller than the DNA used to stimulate the reaction; there is no evidence of covalent attachment of the product to the template.

Response to Various DNA Templates

Table 4 compares the response of the AMV DNA–DNA polymerase to different DNA templates. Unlike the thoroughly studied DNA-dependent DNA polymerase

Table 3. PROPERTIES OF DNA-DIRECTED PRODUCT OF THE AMV POLYMERASE

Treatment	TCA precipitable c.p.m.	TCA precipitable Per cent
None	810	100
Ribonuclease (50 μg/ml.)	785	97
KOH (0·33 M, 18 h)	773	95
Deoxyribonuclease (120 μg/ml.)	177	22
Spleen phosphodiesterase (0·28 units/ml.)	14	1·7

The product DNA made on double-stranded MEF DNA template was synthesized using the standard incubation mixture on a 1 ml. scale. The specific activity of the ^{3}H-ATP used was 4,000 c.p.m./pmole. After reaction at 37° C for 2 h, the product was isolated by making the reaction mixture 0·4 M with respect to NaCl and 1 per cent sodium dodecyl sulphate (SDS) and extracting for 5 min at room temperature with an equal volume of a mixture of phenol–cresol (10 : 1). The aqueous phase was filtered through a 'Sephadex G-50' column (0·9 × 100 cm) and the purified material precipitated with alcohol. It was then dissolved in 0·01 M Tris–0·1 M NaCl (*p*H 8·3) (TN buffer) and portions of this solution were used for the different experiments. The digestion with ribonuclease was carried out in 0·5 ml. TN buffer for 90 min at 37° C. Digestion with deoxyribonuclease was for 3 h at 37° C in TN buffer plus 0·005 M $MgCl_2$. The reaction with spleen phosphodiesterase was in 0·02 M ammonium acetate buffer (*p*H 6·0) at 37° C for 2·5 h in 0·1 ml. The alkali treatment was in 20 μl. in 0·33 M KOH for 18 h at 37° C.

of Kornberg[4], these oncogenic viral DNA polymerases prefer double to single-stranded DNA. A particularly interesting example is the f1 DNA, a single strand DNA quite similar to that found in the DNA bacteriophage φX174 which has been shown[5] to be an excellent template for the Kornberg enzyme. We have seen no signs of template function even in extensive incubations with f1 DNA. The f1-RF DNA, a covalently linked double-stranded circular DNA, is also inactive. This is not surprising because at least one of the two strands would have to be nicked before replication could commence.

Table 4. RESPONSE OF AMV DNA–DNA POLYMERASE TO VARIOUS DNA TEMPLATES

DNA	c.p.m. ^{3}H-TTP incorporated None	Double-stranded	Single-stranded or denatured
E. coli	—	1,006	239
MEF	135	638	135
CEF	76	889	403
T6	120	2,320	460
f1	50	—	19
f1-RF	50	128	—

The reactions were in the standard conditions and processed as in Table 1. The ^{3}H-dATP had a specific activity of 350 c.p.m./pmole. Incubations were for 30 min at 37° C. DNA concentrations were about 3 μg/0·25 ml.

Proof by Hybridization

The response to quite disparate DNA templates provides an excellent opportunity of deciding by molecular hybridization whether the DNA added is in fact serving as an instructive agent in the polymerization. The required DNA–DNA hybridization can readily be performed using the Denhardt[6] modification of the Gillespie and Spiegelman[7] method for hybridizing RNA to DNA fixed to membrane filters. We have found (Horowitz and S.S., unpublished observations) that including the

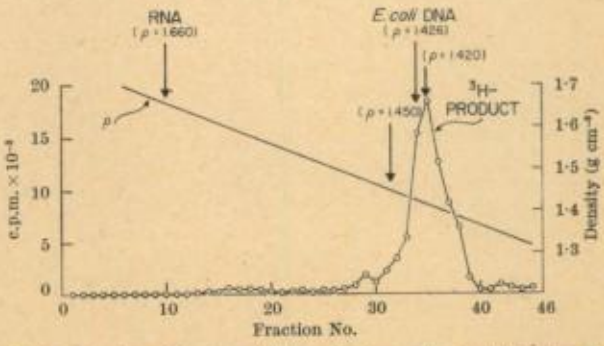

Fig. 2. Caesium sulphate equilibrium density gradient centrifugation of AMV DNA–DNA polymerase product. A 1 ml. standard reaction (Table 1) containing 133 μg viral protein, 9 μg MEF DNA, and ^{3}H-dATP at $4·1 \times 10^3$ c.p.m./pmole was incubated for 2 h at 37° C. The reaction mixture was deproteinized by phenol extraction at room temperature in the presence of 0·4 M NaCl and SDS 1 per cent final concentration. An aliquot of the purified product was mixed with saturated Cs_2SO_4 to a density of 1·550 and centrifuged at 33,000 r.p.m. at 20° C for 60 h in a Spinco 'SW-56' rotor. *E. coli* DNA (ρ = 1·426) and ^{32}P-labelled 18S ribosomal RNA (ρ = 1·660) were used as internal markers. After centrifugation, fractions were collected from the bottom of the tube and processed as in Table 1.

alkaline wash used in the procedure of Warnaar and Cohen[8] yields acceptable backgrounds.

Table 5 shows the results of hybridizing each of three DNA products to the three DNA templates used in the synthetic reactions. The data are clear cut: the hybridizability of each product is much superior when challenged with the DNA actually used in its synthesis. The percentages of the input counts hybridized in the homologous hybridizations of Table 5 are quite considerable: 10, 16 and 59 per cent for the mouse, *E. coli* and T6, respectively. For the mouse the value is surprisingly high in view of the complexity of the genome. We have obtained similar results with chicken DNA. Such data suggest that the DNA polymerase of the oncogenic viruses is not copying random segments of these vertebrate DNAs. Clearly it would be desirable to identify the components of the genome that are being chosen selectively for amplification.

Another feature of the hybridization experiments is exhibited in Table 6 which examines the effect of denaturation on the ability of the synthesized DBA to hybridize to its homologous templates. Denaturation strikingly augments the amount of product available for hybrid formation. The results suggest therefore that much (70–80 per cent) of the DNA synthesized is in the double-stranded state.

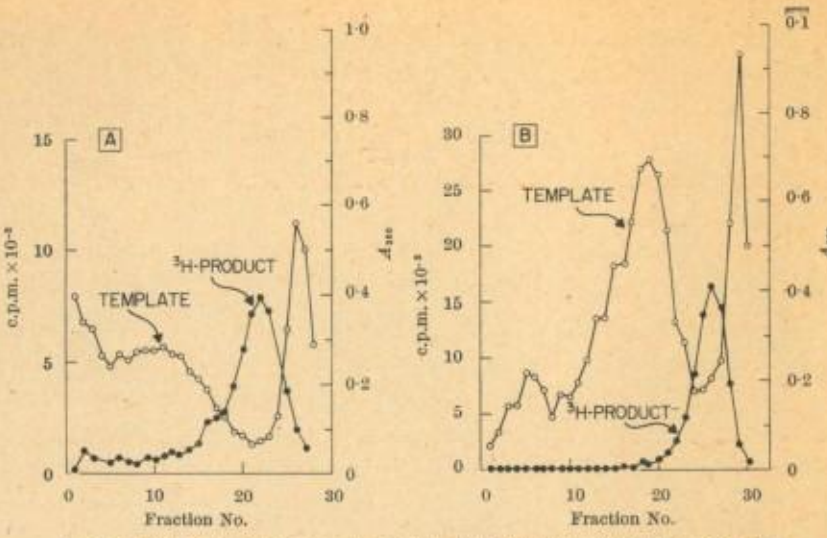

Fig. 3. Neutral and alkaline sucrose gradient centrifugation of AMV DNA–DNA polymerase product. A 1 ml. reaction containing 133 μg of AMV protein pretreated as described in Fig. 1, 9 μg of *E. coli* DNA, and ^{3}H-dATP (4×10^3 c.p.m./pmole) was incubated for 2 h at 37° C and the product purified as described in Fig. 2. 180 μg of the *E. coli* DNA used as template and an aliquot of the product of the reaction were layered on (*A*) neutral (8–22 per cent sucrose–0·01 M Tris (*p*H 8·3)–0·25 M NaCl) or (*B*) alkaline (8–22 per cent sucrose–0·25 M NaOH (*p*H 12·4)–0·05 M EDTA) sucrose gradients. Each gradient was prepared on a 0·5 ml. cushion of 70 per cent sucrose. Centrifugation was at 40,000 r.p.m. and 4° C for 5·5 h in the Spinco 'SW-41' rotor. Fractions were collected from the bottom of the tube and processed as in Table 1.

Conclusions

While eliminating the resident RNA template an opportunity is presented to examine the acceptability of other RNA molecules by the RNA-directed polymerase. Experiments along these lines will be reported later.

The fact that the DNA-directed polymerases described here readily accept—indeed prefer—double-stranded DNA as templates distinguishes them from the DNA-dependent DNA polymerases so far reported. If the oncogenic viral polymerases are in fact true replicases, they should initiate chains, a feature that can be examined in syntheses with γ-^{32}P-labelled deoxyribonucleotides.

All the six oncogenic RNA viruses which we have examined possess the DNA-directed polymerase, but this activity could not be detected in five non-oncogenic viruses: reo, polio, influenza, vesicular stomatitis and Newcastle disease viruses.

It is now clear that RNA oncogenic viruses contain two DNA polymerase activities: one which uses single-stranded RNA as a template and in the process generates a DNA–RNA hybrid; and a second, described here, which accepts double-stranded DNA as a template and yields a principally double-stranded product. The primary function of the latter may be to amplify the oncogenic DNA duplex once it is formed. The multiple copies so produced could markedly increase the probability of a successful integration. It is, of course, still necessary to clarify how the DNA–RNA hybrid is converted to the DNA–DNA duplex, a necessary reaction which we believe is occurring in our preparations. Is a third catalytic function required or can one of the two activities we have already identified mediate this step? The use of suitably constructed DNA–RNA structures should resolve this problem. It will be necessary to purify the relevant proteins and tackle the formidable problem of the logistics of preparing purified viruses in amounts adequate for a realistic attempt at this kind of enzymology.

Table 5. HYBRIDIZATION OF DNA-DIRECTED PRODUCTS TO VARIOUS DNAs

DNA on filter	Template for synthetic DNA: Mouse	*E. coli*	T6
Mouse	*8,698* (100%)	587 (6%)	1,477 (2%)
E. coli	454 (5%)	*7,724* (100%)	197 (0·4%)
T6	152 (2%)	168 (2%)	*54,673* (100%)

Radioactive material in each hybridization was equivalent to $3{\cdot}3 \times 10^4$, $4{\cdot}9 \times 10^4$ and $9{\cdot}4 \times 10^4$ c.p.m. for DNAs synthesized with mouse, *E. coli*, and T6 DNA, respectively. The numbers represent c.p.m. found per 150 μg of DNA on the filter; those in parentheses represent percentage of that observed in the homologous hybridization. Products were prepared as described in Table 3, and dissolved in 0·5 ml. 0·07 × SSC (1 × SSC = 0·15 M NaCl – 0·015 M sodium citrate, *p*H 7). The hybridization reactions were carried out using DNA immobilized on membrane filters and product DNA in solution in 200 μl. of 3 × SSC at 66° C for 12 h. For denaturation, 100 μl. of the product was made 0·1 M with respect to KOH and kept at room temperature for 10 min. The solution was then neutralized with an equivalent amount of HCl and diluted to a final volume of 200 μl. with a calculated amount of SSC solution so that the final solution was 3 × SSC. The filters were loaded with 100–150 μg of denatured DNA, allowed to dry at room temperature, and then transferred to a vacuum oven at 80° C for 2 h at a pressure of 25 mm Hg. The filters were pre-incubated in Denhardt[9] solution (0·02 per cent bovine serum albumin, 0·02 per cent 'Ficoll' and 0·02 per cent polypyrrolidene in 3 × SSC) at 66° C for 6 h immediately preceding hybridization. After hybridization for 12 h. the filters were taken out; each was washed on either side with 100 ml. of 10^{-3} Tris (*p*H 9·3), dried and counted. C.p.m. fixed in homologous hybridizations are italicized.

Table 6. HYBRIDIZATIONS OF NATIVE AND DENATURED PRODUCT DNA WITH TEMPLATE DNA

DNA	c.p.m. Native	Denatured
Mouse	2,878	8,698
E. coli	2,098	7,724
T6	10,834	54,673

The same products and conditions are used here as for the experiment of Table 5. The "native" was not, however, subjected to the alkaline treatment as in the legend of Table 5.

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