Formation of Viral RNA–DNA Hybrid Molecules by the DNA Polymerase of Sarcoma-Leukaemia Viruses

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Viral RNA is the template for the DNA polymerase of the sarcoma-leukaemia viruses: a "natural" RNA-DNA hybrid is formed by the viral DNA polymerase, and a "synthetic" RNA-DNA hybrid is formed by annealing viral RNA with the DNA product of the DNA polymerase reaction.

We have postulated that RNA tumour viruses possess a DNA polymerase dependent on viral RNA (ref. 1) and several laboratories have recently reported that leukaemia and sarcoma viruses of the mouse, chicken and cat incorporate radioactive deoxyribonucleotides into an acid-insoluble form[2–5]. As well as showing that information can flow from RNA to DNA, this system may provide unique information for the understanding of carcinogenesis. Although it is clear that DNA is the reaction product, the evidence that viral RNA is the template has been indirect; pretreatment of virus particles with large amounts of ribonuclease inhibits subsequent enzyme activity; RNA tumour viruses contain a 70S RNA component but little DNA.

We describe here two lines of evidence demonstrating that viral RNA is the template for the murine sarcoma virus (MSV) DNA polymerase: first, viral RNA–DNA hybrid molecules are formed by the MSV DNA polymerase and second, the DNA product formed by the MSV DNA polymerase hybridizes with viral RNA.

Viral RNA–DNA Hybrids

We have characterized previously the DNA polymerase present in MSV (ref. 4). The standard assay is conducted in a final volume of 100 μl. containing 40 mM Tris buffer (*p*H 8·1), 30 mM NaCl, 5 mM dithiothreitol, 2·5 mM $MgCl_2$, 0·01 per cent 'Nonidet P40', 0·1 mM dATP, dGTP, dCTP, and 10 μCi of ^{3}H-TTP (11·4 Ci/mmole). Enzyme activity is linear with time for at least 90 min and is proportional to virus concentration from at least 2 to 20 μg of viral protein per 100 μl. All four deoxyribonucleotides are incorporated into an acid-insoluble, alkali-stable, deoxyribonuclease-digestible product.

To determine whether a viral RNA–DNA hybrid is synthesized by the viral DNA polymerase, MSV(M) containing ^{32}P-labelled viral RNA was used in the standard enzyme assay with ^{3}H-TTP as the labelled deoxyribonucleotide precursor of DNA. The reaction was stopped after 0, 15, 30, 60, and 90 min and the products were analysed by zone sedimentation (Fig. 1). In the conditions used, ^{32}P-labelled viral RNA sediments about two-thirds down the tube. No significant amount of ^{3}H-labelled material from the zero time reaction sediments in this 70S region, but products of increasing times of reaction include increasing amounts of ^{3}H-DNA co-sedimenting with 70S RNA. At 90 min, more than 8,000 c.p.m. of the ^{3}H-DNA product from 50 μl. of reaction mixture co-sediments with 70S RNA.

The peak fraction containing both 70S ^{32}P-RNA and ^{3}H-DNA were pooled as a "hybrid fraction" for further study. The hybrid fraction was treated with pronase (125 μg/ml. for 1 h at 37° C) and recentrifuged in a sucrose density gradient. Again the ^{3}H-DNA and ^{32}P-RNA sedimented in the same 70S position, indicating that the DNA and RNA were not held together by a protein link. Stronger evidence for the hybrid nature of the product

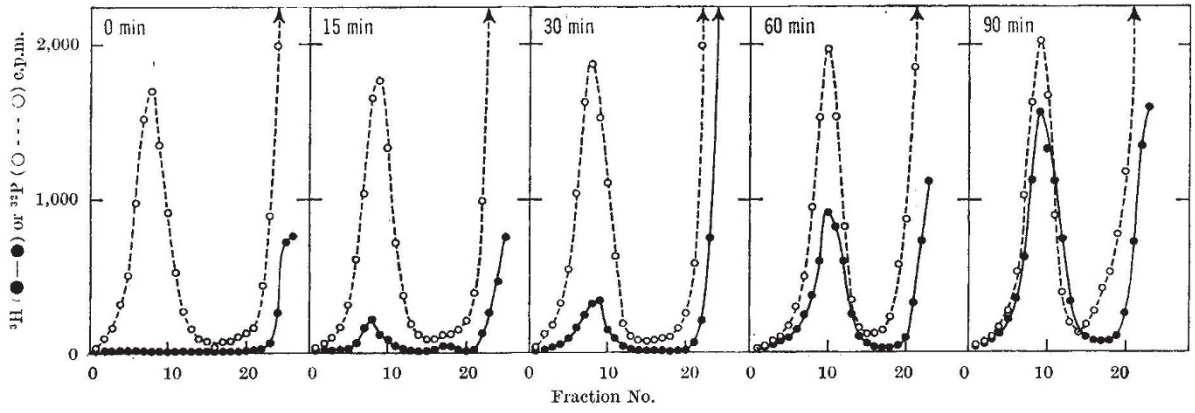

Fig. 1. Zone sedimentation of the product of the MSV DNA polymerase reaction. The Moloney isolate of murine sarcoma virus, MSV(M), purified from the growth medium of the MSV(M) transformed rat cell line, 78A1[4], was used in these experiments. ^{32}P-labelled MSV(M) was purified from the media[4] of fifty 78A1 monolayer cultures (250 ml. Falcon plastic) grown for 24 h in Eagle's minimal essential medium (containing one-fourth the normal phosphate concentration) with 10 per cent foetal calf serum containing 50 μCi/ml. of carrier-free $^{32}PO_4^{3-}$. Purified virus was dialysed for 3 h against 0·01 M Tris-HCl (*p*H 8·1). Five 200 μl. amounts of virus (34·5 μg of protein) were made up to 500 μl. containing the standard assay mixture with 50 μCi of ^{3}H-TTP. At 0, 15, 30, 60 and 90 min of incubation at 37° C, one assay tube was removed, and the reaction stopped by the addition of 10 mM EDTA and 0·5 per cent SDS. The preparations were layered over 11·4 ml. linear 15–30 per cent sucrose gradients in 0·01 M Tris-HCl (*p*H 7·4)—0·1 M NaCl—0·001 M EDTA, and centrifuged at 4° C for 3·5 h in the Spinco 'SW41' rotor at 36,000 r.p.m. Fractions (0·5 ml.) were collected and 50 μl. aliquots plus 100 μg of carrier calf thymus DNA were precipitated with 0·6 M trichloroacetic acid and collected on membrane filters. The acid-insoluble ^{3}H and ^{32}P c.p.m. were determined in a Beckman liquid scintillation spectrometer. (^{32}P c.p.m. at the top of the gradient represent viral envelope phospholipid and 4–5S RNA present in tumour viruses. ^{3}H c.p.m. at the top are mostly ^{3}H-TTP from the assay mixture and also some free ^{3}H-DNA product.) ○ - - - ○, Viral ^{32}P-RNA; ●—●, ^{3}H-DNA product.

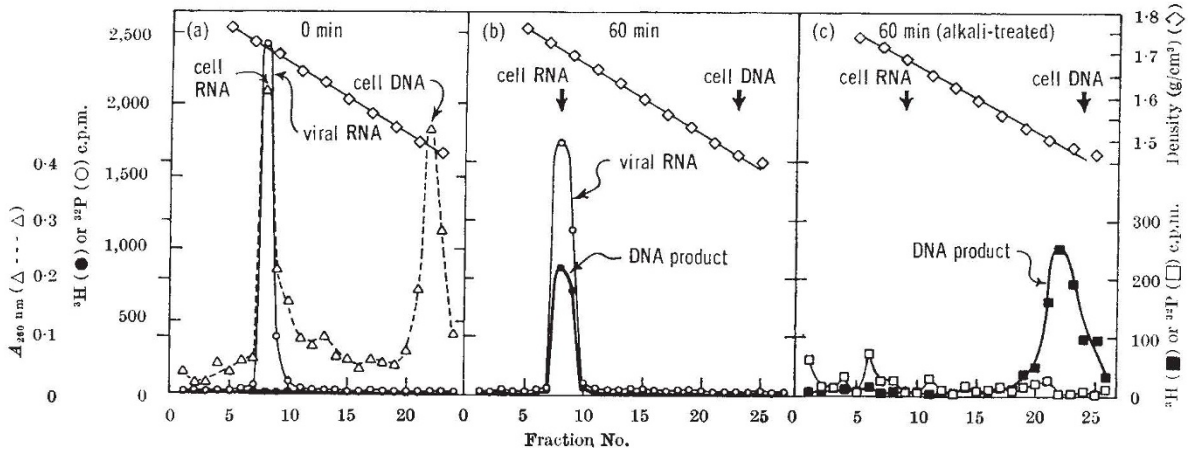

Fig. 2. Equilibrium sedimentation of the viral RNA–DNA hybrid fractions in Cs_2SO_4 density gradients. The peak fractions containing ^{3}H-DNA and/or viral ^{32}P-RNA after zone sedimentation of the 0 and 60 min reaction products (Fig. 1) were pooled and dialysed against 0·1 × SSC (SSC = 0·15 M NaCl–0·015 M sodium citrate). Two hundred μl. of hybrid and 20 μg each of mouse cell DNA and RNA (absorbance markers) were made up to a final volume of 3·1 ml. in 2 × SSC, 2·60 g of Cs_2SO_4 (Harshaw Chemical Co.) were added, and the tubes were centrifuged at 20° C for 84 h in the Spinco 'SW39' rotor at 33,000 r.p.m. Fractions (0·17 ml.) were collected and the refractive index, absorbance at 260 nm, and acid-precipitable radioactivity determined.

was obtained using equilibrium band sedimentation in a Cs_2SO_4 gradient which resolves DNA from RNA by density. As shown in Fig. 2*a*, the 70S ^{32}P-RNA band is at a density close to that of the cell RNA marker ($\rho \simeq 1{\cdot}68$ g cm^{-3}) and distinct from that of the cell DNA marker ($\rho \simeq 1{\cdot}44$ g cm^{-3}). But the 60 min ^{3}H-DNA band is coincident with viral ^{32}P-RNA (Fig. 2*b*). Treatment of the hybrid fraction with 0·2 M NaOH for 20 min at 80° C before density gradient analysis—which would hydrolyse RNA—lowers the density of the ^{3}H-DNA component to that of free DNA (Fig. 2*c*). Three additional hybrid fractions were analysed with similar results. We conclude: (1) that the immediate product of the MSV DNA polymerase reaction is a viral RNA–DNA hybrid; and (2) that only a small portion of each RNA molecule seems to participate in hybrid duplexes, for the buoyant density of the hybrid is close to that of viral RNA.

The RNA–^{3}H-DNA fractions isolated by zone sedimentation of reaction mixtures after 30, 60 and 90 min were sedimented through alkaline sucrose gradients which free polynucleotide chains from one another. After 10 h at 36,000 r.p.m. in the 'SW41' rotor (Fig. 3), ^{14}C-DNA released from marker adenovirus sediments at 34S near the bottom of the tube. The alkali-released ^{3}H-DNA from the hybrid sediments close to 7S in six determinations. This corresponds to a molecular weight of single-stranded DNA of about 200,000 daltons (Table 1).

Table 1. SIZE OF DNA IN VIRAL RNA–DNA HYBRID

Incubation (min)	Sedimentation coefficient in alkali*	Mol. wt.†
30	6·3	155,000
30	5·9	150,000
60	6·7	181,000
60	6·7	181,000
90	7·7	255,000
90	6·8	188,000

* Sedimentation coefficient in alkali determined in Fig. 3.
†Molecular weight of single stranded DNA calculated from S value as described by Studier[6].

Further evidence that viral RNA is the template for the MSV DNA polymerase was obtained by annealing viral 70S ^{32}P-RNA with enzymatically synthesized ^{3}H-DNA and analysing the product by equilibrium sedimentation in Cs_2SO_4 density gradients. When viral RNA and DNA are mixed but not annealed, the viral DNA band is at the density of unhybridized DNA (Fig. 4*a*). But the incubation of viral ^{32}P-RNA with ^{3}H-DNA in annealing conditions converts viral DNA to a hybrid—it again coincides with viral RNA (Fig. 4*b*).

Does the Virus contain DNA?

RNA tumour viruses are assumed to contain only RNA but because DNA might serve as a template for the viral DNA polymerase, we attempted to determine whether MSV(M) contains appreciable amounts of DNA. We isolated MSV(M) from the media of 78A1 cells grown for 24 h in the presence of 2 mCi of ^{3}H-thymidine; the purified preparation represented 2·21 mg of protein and 41,840 c.p.m. (^{3}H). Thus less than 120,000 daltons of DNA are present per virus particle (on the basis of the specific radioactivity of newly synthesized cell DNA and an assumed virus particle content of 60 per cent protein and 2 per cent RNA and a particle weight of 450×10^6 daltons[7,8]).

MSV (^{3}H-thymidine pre-labelled) was used in the standard enzyme assay with ^{32}P-TTP as the labelled deoxyribonucleotide precursor of DNA. The reaction mixture was treated with 0·5 per cent sodium dodecyl sulphate and analysed by zone sedimentation. Most of the

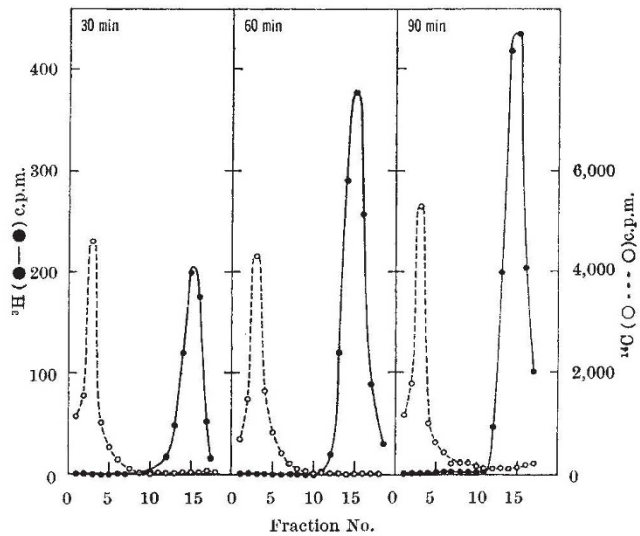

Fig. 3. Zone sedimentation of ^{3}H-DNA from RNA–^{3}H-DNA hybrids in alkaline sucrose density gradients. The 30 min (200 μl.), 60 min (150 μl.), and 90 min (100 μl.) hybrid fraction, isolated as described in Fig. 1, plus 3 μl. of ^{14}C-thymidine labelled adenovirus type 7 were layered on 11·3 ml. linear 5–20 per cent sucrose gradients in 0·3 M NaOH–0·7 M NaCl–0·001 M EDTA, and centrifuged at 4° C for 10 h in the Spinco 'SW41' rotor at 36,000 r.p.m. Fractions (0·75 ml.) were collected and the acid-insoluble, alkali-stable radioactivity was determined[4]. ○ - - - ○, Adenovirus DNA marker; ●—●, DNA product from RNA–DNA hybrid.

^{32}P-DNA product sedimented to the position of the RNA–DNA hybrid but ^{3}H c.p.m. from pre-labelled virus did not co-sediment with the DNA product. Thus the ^{3}H-thymidine-labelled material in MSV does not seem to participate in the formation of the ^{3}H-DNA-containing hybrid. The small amount of ^{3}H-thymidine present in the virus preparation may represent DNA in structures contaminating the virus preparation, or possibly the natural DNA product of the viral DNA polymerase.

Significance of the DNA Product

Our experiments strongly argue that viral RNA is the template for the DNA polymerase present in the murine sarcoma virus for (1) a "natural" viral RNA–DNA hybrid is formed during the enzymatic reaction, and (2) a "synthetic" RNA–DNA hybrid is formed by annealing the DNA product with viral RNA. The viral RNA–DNA hybrid is probably the initial product of the MSV DNA polymerase reaction for only small amounts of free DNA product are found even after 90 min of reaction.

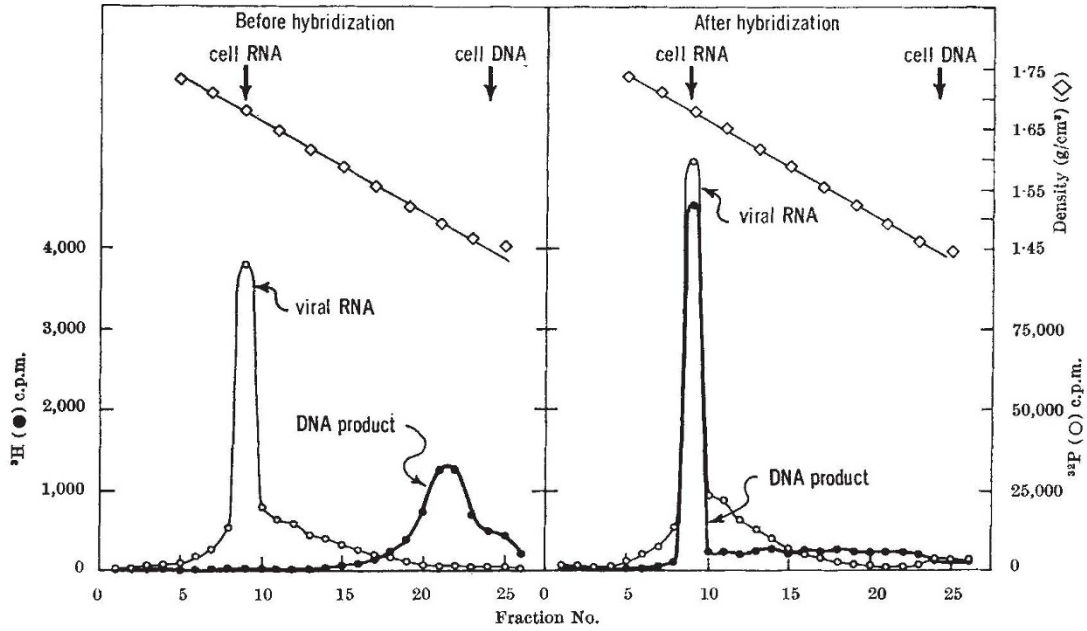

Fig. 4. Hybridization of viral ^{32}P-RNA with the ^{3}H-DNA product of the MSV DNA polymerase. Viral ^{32}P-RNA was prepared from ^{32}P-labelled MSV(M) and ^{3}H-DNA was prepared from a 60 min reaction mixture as follows: after the addition of 0·5 per cent sodium dodecyl sulphate, the preparations were extracted three times with phenol saturated with 0·1 M NaCl–0·01 M Tris–HCl (*p*H 7·4)–0·001 M EDTA. The aqueous layer was dialysed against 0·1 × SSC. The ^{3}H-DNA preparation was treated with 0·2 N NaOH at 80° C for 20 min and neutralized to denature the DNA and destroy unlabelled viral RNA. Denatured ^{3}H-DNA (4,400 c.p.m.) and 5·8 μg of viral ^{32}P-RNA (40,500 c.p.m./μg) in 400 μl. of 2 × SSC were annealed at 60° C for 9 h. An identical mixture of ^{3}H-DNA and ^{32}P-RNA, not annealed, served as a control. Both samples were centrifuged to equilibrium in Cs_2SO_4 gradients and processed as described in Fig. 2.

The DNA component in an individual hybrid duplex moves at 7S, corresponding to a molecular weight of 200,000. A similar value is obtained for the average amount of DNA formed per virus particle in a 90 min enzyme reaction (see Fig. 1, calculated from 3·45 μg of viral protein per 50 μl. reaction mixture, 8,000 c.p.m. incorporated into DNA, assuming equimolar amounts of A, G, C and T in the DNA product and a 60 per cent protein content and 450×10^{6} particle weight for the virus[7,8]). These findings confirm the correspondence between the RNA–DNA hybrid and the free 70S viral RNA: apparently only a small fraction of each RNA molecule is in hybrid form.

Further experiments must resolve whether (1) the complete viral genome is transcribed to DNA *in vivo* or (2) the production of a specific short piece(s) of DNA is a true function of the polymerase. According to the first alternative, the hybrid may be an intermediate in the replication of virus or in the production of transforming DNA, but the failure of the *in vitro* enzyme reaction to produce hybrid structures longer than about 2–7 per cent of the viral genome would then have to be explained. Nuclease activities might degrade the rest of the chain formed *in vitro*, or perhaps host cell or viral induced gene products are required to transcribe the entire viral genome into DNA. The second alternative opens up several possibilities: this piece(s) of DNA may, for example, code for critical protein(s) in viral development. The distinction between these two possibilities is especially important, for the role of viral DNA appears, on the basis of inhibitor studies, to be essential for both viral replication and cell transformation[1]. The mechanism of carcinogenesis by sarcoma–leukaemia viruses may even provide a model for possible reversal of RNA transcription during normal cell function, such as the intriguing expression of RNA tumour virus genetic information by cellular genes during embryonic development in the mouse[10].

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