TRANSCRIPTION OF HETEROPOLYMERIC REGIONS OF AVIAN MYELOBLASTOSIS VIRUS HIGH MOLECULAR WEIGHT RNA WITH ESCHERICHIA COLI DNA POLYMERASE I

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SUMMARY: High concentrations of a purified preparation of Escherichia coli DNA polymerase I are able to transcribe heteropolymeric regions of avian myeloblastosis virus high molecular weight RNA, both in the presence and absence of oligo (dT) as a primer. Some of the DNA transcripts specifically hybridize to avian myeloblastosis virus high molecular weight RNA. The size of the DNA transcript is approximately 5S. In contrast to the reverse transcriptase from RNA tumor viruses and human acute leukemic cells, transcription of poly(A) regions of viral high molecular weight RNA into poly (dT) and poly (dA) is 5 to 40-fold greater than transcription of heteropolymeric regions of this RNA. Escherichia coli DNA polymerase is 10 to 200-fold less efficient in transcription of heteropolymeric regions of avian myeloblastosis virus high molecular weight RNA relative to activated salmon sperm DNA than DNA polymerases from avian myeloblastosis virus, mammalian type-C viruses, and human acute leukemic cells.

One characteristic property of the DNA polymerase (reverse transcriptase) from RNA tumor viruses is the ability to make DNA copies complementary to heteropolymeric regions of a natural RNA. This property has been amply demonstrated with reverse transcriptases from known viruses, especially from avian myeloblastosis virus (AMV) (1-3) and from human acute leukemic cells (4-6). DNA polymerases from uninfected mammalian cells fail to transcribe heteropolymeric regions of single stranded RNA (7-9). However, recently Escherichia coli DNA polymerase I was shown to make DNA copies complementary to ribosomal RNA (10), globin messenger RNA (11), and AMV RNA (12). In earlier studies on the copying of AMV 60-70S RNA (HMW RNA) with E. coli DNA polymerase I, we (2) and others (1,13) reported that under conditions where enzyme activities are normalized for a DNA template-primer, E. coli DNA polymerase I was not capable of synthesizing DNA copies complementary to heteropolymeric regions of

viral HMW RNA. We now report that in the presence of a large excess of the E. coli enzyme it is possible to make DNA copies complementary to heteropolymeric regions of AMV HMW RNA, in addition to the synthesis of poly (dT) and poly (dA). Mammalian DNA polymerases I (7), II (7), and III (8) have not been shown to transcribe heteropolymeric regions of viral RNA, nor for that matter has this been shown with any eukaryotic cell DNA polymerase. Therefore, this property of a DNA polymerase found in animal cells still appears to strongly indicate that the polymerase in question is a viral-like reverse transcriptase. However, it is only one of several criteria needed to establish that a polymerase isolated from cells rather than from a known RNA tumor virus is probably derived from an RNA tumor virus or is a product of viral gene expression (14-18).

Materials and Methods: A purified preparation of E. coli DNA polymerase I was a gift of Dr. L. Loeb. Avian myeloblastosis virus was generously supplied by Dr. Joseph Beard (Duke University). Viral RNA was isolated as previously described (5). For DNA·RNA hybridization studies, radiolabelled DNA was synthesized as described in the legend to table 1, and the reaction was terminated by the addition of SDS to 1% and NaCl to 0.12 M. DNA products were then purified by extraction with phenol-cresol and CTAB precipitation as described earlier (4,19), treated with 0.3 N KOH (95°, 10 minutes) to hydrolyze template RNA, then neutralized with HCl. The annealing reaction mixture contained 1500-3000 cpm ^{3}H-DNA and 1-2 μg viral RNA in 0.12 ml of 50% formamide, 0.45 M NaCl, 0.045 M sodium citrate buffer (pH 7), 1 mM diethyloxydiformate (Eastman) and incubated for 48 hrs. at 37°C. Cesium sulfate (Schwarz-Mann) equilibrium density gradient analysis was performed as described before (4,19).

Results:

AMV HMW RNA as a Template: Figure 1A shows the incorporation of radiolabelled dCTP into DNA with various concentrations of AMV HMW RNA. Addition of oligo (dT) stimulates the reaction approximately ten fold as shown by the incorporation of [^{3}H]dAMP into DNA (Fig. 1B). The values for the incorporation of all four labelled deoxyribonucleoside triphosphates into DNA are summarized in Table 1.

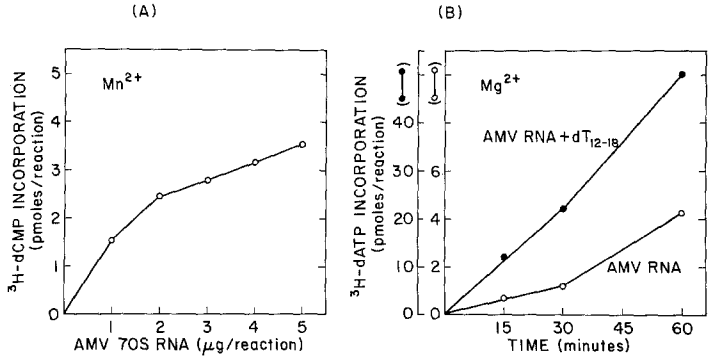

Figure 1 (A). DNA synthesis with E. coli DNA polymerase I as a function of AMV HMW RNA concentration.

Assays were carried out in the presence of Mn^{++} under standard reaction conditions as outlined in legend to table 1 except AMV HMW RNA concentration was varied between 1 and 5 μg per reaction. The specific activity of [^{3}H]dCTP used was 9000 cpm/pmole.

Figure 1 (B). DNA synthesis with E. coli DNA polymerase I and AMV HMW RNA in the absence and presence of oligo(dT) as a primer.

Assays were carried out in a standard reaction mixture containing 5 mM $MgCl_2$ and 0.5 μg of $(dT)_{12-18}$. Specific activity of [^{3}H]dATP used was 5625 cpm/pmole.

The incorporation of dAMP and TMP into DNA is much greater than the incorporation of dCMP and dGMP in the presence of either magnesium or manganese, showing that synthesis of DNA is predominantly homopolymeric, poly (dT) and poly (dA)·poly (dT). Similar synthesis of poly (dT) and poly (dA)·poly (dT) has been observed when Rous sarcoma virus was used as a template-primer with E. coli DNA polymerase I (13). As before, oligo (dT) stimulates the incorporation of dAMP; the table also shows that TMP incorporation is similarly enhanced whereas no such stimulation of the incorporation of dCMP or dGMP into DNA is observed. These results suggest that in the presence of oligo (dT) as a primer, an increase in poly(A) transcription occurs and the resultant poly (dT) in turn acts as a template for the synthesis of poly (dA). Therefore, even under conditions which allow detectable heteropolymeric DNA synthesis, the predominant reaction is still homopolymer synthesis.

Table 1

DNA Synthesis with E. coli DNA Polymerase I and AMV HMW RNA

	pmoles/μg enzyme			
Reaction Conditions	[^{3}H]dATP	[^{3}H]dCTP	[^{3}H]dGTP	[^{3}H]TTP
A. Mg^{2+}				
Complete	3.25	0.49	0.27	1.55
Complete + $(dT)_{12-18}$ (4μg/ml)	8.2	0.24	0.22	2.42
Complete + $(dT)_{12-18}$ (10μg/ml)	50.4	0.55	0.18	48.2
B. Mn^{2+}				
Complete	13.1	1.05	0.34	6.74
Complete + $(dT)_{12-18}$ (4μg/ml)	15.2	0.51	0.34	121.0
Complete + $(dT)_{12-18}$ (10μg/ml)	110.6	0.35	0.13	22.3

DNA polymerase assays were carried out at 37°C for 1 hr. in a standard reaction mixture (0.05 ml) which contained: 65mM Tris-HCl (pH 8), 5 mM $MgCl_2$ (or 0.5 mM $MnCl_2$), 8 mM dithiothreitol, 80 μM each of three unlabelled deoxyribonucleoside triphosphates and one labelled deoxyribonucleoside triphosphate (10 μM). The labelled deoxyribonucleoside triphosphates had the following specific activities: [^{3}H]dATP (5625 cpm/pmole), [^{3}H]dCTP (9,000 cpm/pmole), [^{3}H]dGTP (3150 cpm/pmole), and [^{3}H]TTP (8100 cpm/pmole). Each reaction mixture contained 3.2 μg of E. coli DNA polymerase I and 2 μg of AMV HMW RNA. The reaction was stopped by the addition of 50 μg yeast tRNA and 2 ml of 10% trichloracetic acid containing 0.02M sodium pyrophosphate, collected on millipore filters and counted (4). Radiolabelled DNA products for hybridization studies were prepared in the presence of 100 μg/ml of actinomycin D.

This is in contrast to the results obtained with AMV HMW RNA directed reaction of reverse transcriptase from AMV and human acute leukemic cells where all four deoxyribonucleoside triphosphates are incorporated with about equal

Table 2

Incorporation of Deoxyribonucleoside Monophosphates into DNA Synthesized with AMV HMW RNA as a Template-Primer with Various DNA Polymerases

	Source of DNA Polymerase		
Labelled Substrate	AMV	Human Acute Leukemia*	E. coli
[^{3}H]dCTP	1.39	0.74	1.81 (3.08)
[^{3}H]dGTP	1.00	1.00	1.00 (1.00)
[^{3}H]dATP	1.03	1.15	12.04 (38.5)
[^{3}H]TTP	1.11	0.79	5.74 (19.8)

Values of deoxyribonucleoside monophosphate incorporation are calculated using the incorporation of dGMP as 1.00 in reactions carried out in the presence of Mg^{++}. Values in parenthesis represent numbers obtained from reactions carried out in the presence of Mn^{++}.

* This DNA polymerase was isolated from a cytoplasmic pellet (ρ=1.16 g cm^{-3}) obtained from human acute leukemic cells and has both biochemical (4) and immunological (17) properties of type-C virus reverse transcriptase.

efficiency and little or no homopolymer synthesis occurs (3,4 and Table 2). Under most reaction conditions DNA polymerases from uninfected normal mammalian cells do not utilize AMV HMW RNA as a template-primer; however, under certain conditions DNA polymerase II (7) and III (8) of mammalian cells can transcribe poly(A) regions of viral RNA.

Table 3 shows a comparison of the relative response of reverse transcriptases from AMV, Rauscher leukemia virus (RLV), woolly monkey virus, (simian sarcoma virus or SSV-1) and human acute leukemic cells, and of E. coli DNA

Table 3

Template Activity of AMV HMW RNA and Activated Salmon Sperm DNA with Various DNA Polymerases

NA Polymerase	Salmon Sperm DNA (pmoles)	AMV HMW RNA (pmoles)	Ratio of Salmon Sperm DNA Activity / AMV HMW RNA Activity
vian Myeloblastosis Virus	1.2	1.08	1.11
oolly Monkey Virus	9.4	0.59	15.9
auscher Leukemia Virus	8.16	0.40	20.4
luman Acute Leukemia	23.16	0.93	24.9
. coli	99.2	0.43	230.7

eaction conditions were as described in Table 1 using [^{3}H]dCTP as the labelled ubstrate. Activated salmon sperm DNA was prepared as described earlier (2) and as used at a final concentration of 50 µg/ml. Reverse transcriptase from AMV, LV, woolly monkey and human acute leukemic cells were obtained according to arlier procedures (2,4,21).

polymerase I to activated salmon sperm DNA and AMV HMW RNA. AMV reverse transcriptase is the most efficient in transcribing heteropolymeric regions of AMV HMW RNA whereas E. coli DNA polymerase I is least efficient. The preference of E. coli DNA polymerase I for activated DNA as a template is 230 times higher than for AMV HMW RNA as a template whereas AMV reverse transcriptase utilizes both activated DNA and AMV HMW RNA as a template with about equal efficiency. The reverse transcriptase from RLV, SSV-1 and human acute leukemic cells are 10-15 times more efficient in the transcription of AMV HMW RNA relative to activated salmon sperm DNA as a template primer than E. coli DNA polymerase I.

The size of the DNA transcript synthesized from AMV HMW RNA with E. coli DNA polymerase I was determined by analysis of [^{3}H]dCTP labelled DNA product on a sucrose gradient. The sedimentation value is about 5S (Fig. 2),

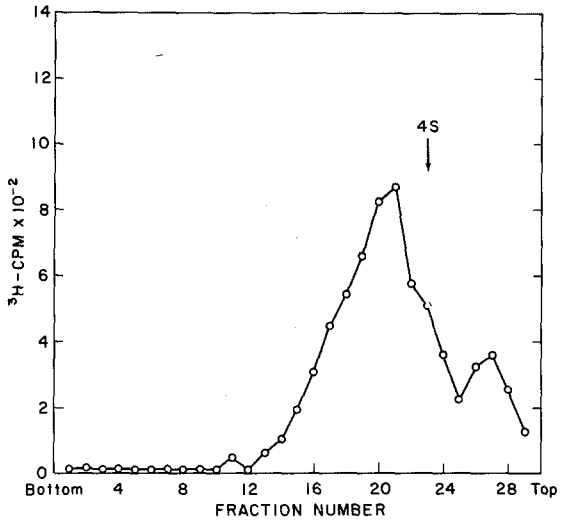

Figure 2. Estimation of the size of the DNA product made with E. coli DNA polymerase I and AMV HMW RNA.

DNA product was prepared in the presence of Mg^{++} as described in legend to table 1, in the absence of oligo(dT). [^{3}H]dCTP was the labelled substrate. The reaction was stopped by the addition of SDS and NaCl, and the nucleic acids were isolated by phenol-cresol extraction as described earlier (4) and heated in 0.3 N KOH at 90°C for 15 min. to hydrolyze the RNA. The alkali treated DNA product was then layered on top of a linear sucrose gradient 5-30% (w/w) in Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.001 M EDTA. The samples were centrifuged for 18 hrs. at 35,000 rpm at 4°C in a spinco SW 50.1 rotor. Three drop fractions were collected from the bottom of the tube and the samples were precipitated with calf thymus DNA and 10% trichloracetic acid, filtered on a millipore filter and counted. Tritiated tRNA was used as an external marker and run simultaneously in a separate gradient.

similar to values obtained for the DNA product synthesized with reverse transcriptase from AMV (3) and human leukemic cells (4,14). The size of the DNA product made with E. coli DNA polymerase I and ribosomal RNA (10) and globin messenger RNA (11) were reported to be about 3S.

Analysis of the DNA product on Cs_2SO_4 equilibrium density gradient. (a) Synthesis of poly (dT) and poly (dA): DNA transcripts labelled with [^{3}H] TTP and [^{3}H] dATP

were analyzed by Cs_2SO_4 equilibrium density gradient centrifugation after annealing with the indicated RNA as described under Materials and Methods. [^{3}H] TTP labelled DNA transcripts consist mainly of poly (dT) as shown by their extensive hybridization to both AMV HMW RNA (Fig. 3A) and poly(A) (Fig. 3B)

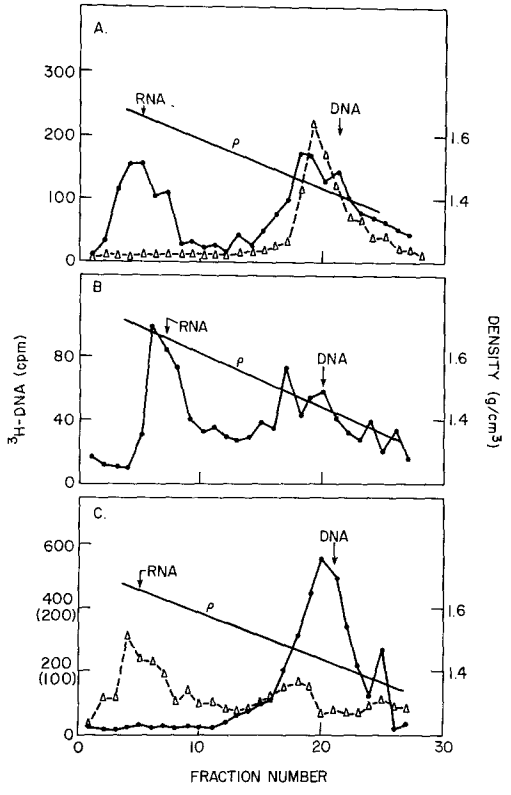

Figure 3. Demonstration of homopolymeric DNA synthesis by E. coli DNA polymerase and AMV HMW RNA.

[^{3}H] DNA was synthesized in a standard reaction of E. coli DNA polymerase I and AMV HMW RNA under conditions (Mg^{++}) described in legend to table 1, using either [^{3}H]TTP or [^{3}H]dATP as the labelled substrate. The product DNA was purified and analyzed on cesium sulfate equilibrium density gradient centrifugation according to an earlier procedure (4). Purified [^{3}H] DNA was then annealed with the indicated RNA as described in Materials and Methods. A. [^{3}H] DNA labelled with [^{3}H]TTP was annealed with 1 µg AMV HMW RNA (●—●), or annealed with 1 µg AMV HMW RNA preannealed with 3 µg poly(dT) (Δ---Δ). B. [^{3}H] DNA labelled with [^{3}H]TTP was annealed with 1 µg poly(A). C. [^{3}H] DNA labelled with [^{3}H]dATP was annealed with 1 µg AMV HMW RNA (●—●) or with 1 µg poly(U) (Δ---Δ)(cpm values in parenthesis). Arrows indicate the positions of internal markers of E. coli 4S RNA and native salmon sperm DNA as determined by absorbance measurements at 260 nm.

and also by their failure to hybridize to AMV HMW RNA which had been prehybridized to unlabelled poly (dT) to mask the poly (A) regions. [^{3}H] dATP labelled DNA hybridizes to poly(U) but not detectably to AMV HMW RNA (Fig. 3C). Therefore, if [^{3}H] TTP or [^{3}H] dATP is used as the labelled substrate, synthesis of labelled heteropolymeric DNA is negligible compared to poly (dT) and poly (dA) synthesis. These results are similar to those obtained with Rous sarcoma virus 70S RNA and E. coli DNA polymerase I (13).

(b) Transcription of Heteropolymeric regions of AMV HMW RNA: As shown in figure 4A the DNA product synthesized from AMV HMW RNA and E. coli DNA polymerase

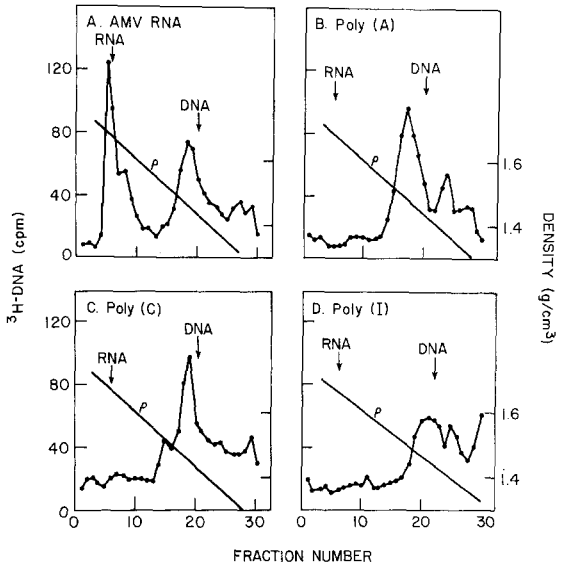

Figure 4. Demonstration of transcription of heteropolymeric regions of AMV HMW RNA by E. coli DNA polymerase I.

[^{3}H]DNA was synthesized and purified from a reaction of E. coli DNA polymerase I with AMV HMW RNA using [^{3}H] dGTP and [^{3}H] dCTP under conditions (Mg^{++}) described in legend to table 1, in the absence of oligo (dT), and analyzed by cesium sulfate equilibrium density gradient centrifugation according to an earlier procedure (4). Purified [^{3}H] DNA was then annealed with the indicated RNA as described in materials and methods. A. [^{3}H] DNA was annealed with 1 μg AMV HMW RNA. B. [^{3}H] DNA was annealed with 1 μg poly(A). C. [^{3}H] DNA was annealed with 1 μg poly(C). D. [^{3}H] DNA was annealed with 1 μg poly(I). Arrows indicate the positions of internal markers of E. coli 4S RNA and native salmon sperm DNA.

I with labelled [^{3}H] dCTP and [^{3}H] dGTP and unlabelled dATP and TTP hybridizes to AMV HMW RNA but not to poly(A) (Fig. 4B), poly(C) (Fig. 4C) or poly(I) (Fig. 4D). These results clearly demonstrate that these DNA transcripts are heteropolymeric, not covalently linked to poly (dT), and do not contain poly (dG) or poly (dC). Analysis of the DNA product labelled with [^{3}H] dCTP and [^{3}H] dGTP synthesized from AMV HMW RNA in the presence of oligo (dT) as a primer also hybridized specifically to AMV HMW RNA (data not shown) and not to poly(A). If oligo (dT) were serving as a primer for the AMV HMW RNA directed reaction, then the labelled heteropolymeric transcript should be covalently attached to unlabelled oligo (dT), and detectable hybridization of the DNA product to poly(A) as well as AMV HMW RNA would be expected. Lack of hybridization of the DNA product with poly(A) indicates that oligo (dT) does not serve as a primer for transcription of heteropolymeric regions of AMV HMW RNA, an observation in contrast to that previously reported with AMV reverse transcriptase (20).

Discussion: Transcription of heteropolymeric regions of RNA tumor virus HMW RNA has so far been a property unique to RNA tumor virus DNA polymerase (1-3, 21) and a DNA polymerase isolated from human acute leukemic cells (4,5). Recently, however, E. coli DNA polymerase I has been shown to transcribe DNA from 28S ribosomal RNA (10), globin mRNA (11), and AMV HMW RNA (12). We also find that in addition to a predominant synthesis of homopolymeric DNA [poly (dT) and poly (dA)] also observed with E. coli DNA polymerase I and globin mRNA (11) and Rous sarcoma virus 70S RNA (13), E. coli DNA polymerase I indeed transcribes heteropolymeric regions of AMV HMW RNA. The relative proportion of heteropolymeric DNA synthesized is only 2-20% of the total DNA product. The heteropolymeric DNA transcript is at least partially complementary to AMV HMW RNA since the DNA product made with AMV HMW RNA template in the presence or absence of oligo (dT) as a primer (labelled with [^{3}H]dCTP and [^{3}H]dGTP) hybridizes to AMV HMW RNA but not to poly(A). Incomplete hybridization of the DNA transcript to the HMW RNA may be due to very small size of some of the DNA transcripts. Oligo(dT) also fails

to stimulate the incorporation of [^{3}H]dCTP or [^{3}H]dGTP. Oligo(dT) is therefore not significantly utilized as a primer for heteropolymeric DNA synthesis. In contrast, an increase of transcription of heteropolymeric regions of AMV HMW RNA in the presence of oligo(dT) as a primer has been reported earlier for AMV reverse transcriptase, and some of the poly(dT) synthesized in the oligo(dT) stimulated reaction is covalently attached to heteropolymeric transcripts (20). In view of the predominance of homopolymeric over heteropolymeric transcription of RNA by E. coli DNA polymerase I, caution should be exercised when this polymerase is utilized instead of viral reverse transcriptase as an enzyme reagent for preparing radiolabelled DNA copies of viral high molecular weight RNA or mRNA.

Compared to the E. coli DNA polymerase I all the reverse transcriptases were relatively more efficient in the transcription of viral HMW RNA when the comparisons were made with enzyme units normalized to activity with a DNA template such as activated salmon sperm DNA. In addition, in contrast to the E. coli enzyme which vastly prefers the copying of poly(A) regions, the reverse transcriptases incorporate all 4 deoxyribonucleotides to approximately the same degree, indicating a preference for transcription of the heteropolymeric regions. The ability of E. coli DNA polymerase I to transcribe heteropolymeric regions of viral HMW RNA does not appear to be shared by mammalian cellular DNA polymerases, which so far have not been shown to transcribe these regions of viral HMW RNA (7-9).

Transcription of heteropolymeric regions of HMW RNA has been used as a criterion for judging whether a DNA polymerase from mammalian cells might be derived from a type-C RNA tumor virus. The capacity of E. coli DNA polymerase I to catalyze this reaction does not undermine this criterion since this enzyme is not generally present in mammalian cells. However, as emphasized elsewhere (4-6,14-17,22,23), a combination of other factors e.g., demonstration of an endogenous RNA-primed RNA-directed DNA synthesis (a true reverse transcription) in a particulate fraction; demonstration that the associated RNA is large and contains virus related sequences; relative response of the partially purified enzyme to certain synthetic template-primers [oligo(dG)·poly(C) and oligo(dT)·poly(A)] in a

manner characteristic of the polymerase of these viruses; size of the partially purified enzyme; and specific immunological relatedness to reverse transcriptase isolated from extracellular RNA tumor viruses is needed in establishing the presence of viral derived or viral related reverse transcriptase activity in cells.

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