

## THE PERTH GROUP

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### A brief review of reverse transcription and reverse transcriptases

#### **Oncoviruses**

In 1911 Peyton Rous excised malignant tumour tissue (a sarcoma) of a Plymouth Rock hen, ground it up using sterile sand, centrifuged the mixture and passed the supernatant through Berkefeld filters (which are impervious to cells including bacteria). When the cell-free filtrates were injected into healthy chickens without tumours many of them developed tumours.<sup>1</sup> Rous' experiments were interpreted without the benefit of electron microscopy which was not invented until 1931. In fact it was not until the 1950s and 1960s that researchers using the electron microscope discovered virus-like particles in the cell-free filtrates. The particles became known as oncoviruses (*oncos*, Greek for tumour) and it has since become standard practice to define "filterable agents" as viruses despite the fact many moieties smaller than bacteria and cells, including molecules, pass through Berkefeld filters.

However, Rous himself expressed doubts that the agents which caused the tumours were infectious in nature. Indeed he warned, "The first tendency will be to regard the self-perpetuating agent active in this sarcoma of the fowl as a minute parasitic organism. Analogy with several infectious diseases of man and the lower animals, caused by ultramicroscopic organisms, gives support to this view of the findings, and at present work is being directed to its experimental verification. But an agency of another sort is not out of the question. It is conceivable that a chemical stimulant, elaborated by the neoplastic cells, might cause the tumour in another host and bring about in consequence a further production of the same stimulant".

#### **Enzymes**

Enzymes are protein catalysts. A catalyst is a substance that increases the rate of a chemical reaction without itself being consumed. At the completion of the reaction the catalyst is chemically unchanged and present in its original amount. A reaction involving an enzyme can be written as:



Within limits the more enzyme present the greater the amount of product formed. The best way to determine the presence and quantity of an enzyme is to take the material in question, extract (isolate) the enzyme (the protein) and measure its mass. This is the only way to determine its properties such as molecular weight, amino acid sequence and optimal conditions for function. However, most often enzymes are measured indirectly, that is, by detecting their product. The amount of product is reported as the enzyme "activity" and will produce unambiguous results if and only if the enzyme in question is the only enzyme present that catalyses that particular reaction.

### **ATPase and oncoviruses**

In the 1920s a molecule, adenosine triphosphate (ATP), was discovered in biological tissue. This molecule is split into adenosine diphosphate (ADP) and phosphate, a reaction facilitated by an enzyme called adenosine triphosphatase (ATPase). (The suffix “ase” is used in biochemistry to denote a protein an enzyme). In the 1960s ATPase was found to be “associated” with an oncovirus (retrovirus). “The ATPase was considered so consistent a component of virions” that is, the virus particles, that it was used to both detect and quantify the number of virus particles. However when it was realized that ATP and ATPases were found in all cells and that its presence in the oncovirus particles depended “upon cell-specific, not virus-specific factors”, the enzyme ceased to be used for the detection and quantification of oncoviral particles.<sup>2</sup>

At the beginning of the 1970s another enzyme called reverse transcriptase became the method of detecting and quantifying oncoviruses. Reverse transcriptase activity played a pivotal role in the isolation (=proof for the existence), detection and quantification of “HIV”.

### **Deoxy- and ribo- nucleic acids**

Cells and viruses are made of several different molecules including proteins and the two nucleic acids DNA and RNA. All three are polymers (Greek *poly* (many) and *meros* (parts)). Proteins are composed of twenty different amino acids and nucleic acids (DNA and RNA) from four nucleotides. Each nucleotide consists of a purine or pyrimidine base, a sugar and phosphoric acid. There are 5 bases: guanine (G), cytosine (C), adenine (A), thymine (T) and uracil (U). Each nucleotide is named after the base it contains. Three of the bases (G, C and A) are common to DNA and RNA. DNA contains T while RNA contains U. The other differences between DNA and RNA are that DNA is double-stranded and RNA single-stranded. In DNA the sugar is deoxyribose while in RNA it is ribose. The opposite (complementary) strands of DNA are held together by hydrogen bonding between opposing bases according to the rule G pairs with C and A with T. A single strand from DNA can pair with RNA according to the rule G pairs with C and A with U. See [HERE](#)

### **Transcription and reverse transcription**

After the discovery of the DNA double helix in 1953 a theory was put forward known as the biological dogma. The theory states that:

1. DNA is self-replicating. There are three main cellular enzymes which catalyse the synthesis of DNA, namely, DNA polymerases  $\alpha$ ,  $\beta$ , and  $\gamma$ .
2. DNA serves as a template for the synthesis of RNA.
3. The transcribed RNA instructs the synthesis of proteins.
4. Proteins are not templates for the synthesis of DNA or RNA.
5. RNA is not a template for the synthesis of DNA.

Under the biological dogma information flows in one direction:  
DNA → RNA → protein.

In 1970 new data broke the dogma. Researchers in the USA predicted and then discovered an enzyme that catalyses the synthesis of DNA using RNA as a template. Since the information DNA → RNA was considered “forwards” transcription, the RNA → DNA is called “reverse” transcription. The enzyme that catalyses this process is known as RNA-directed DNA polymerase or reverse transcriptase. Furthermore, because the process of reverse transcription was discovered in what was claimed to be purified oncoviruses, these viruses then became known as retroviruses. Howard Temin, one of the discoverers of reverse transcriptase, objected to both names. He was uncomfortable about scientists misinterpreting his findings as proof that the enzyme reverse transcriptase and the process of reverse transcription are unique to retroviruses. As Temin predicted, many people did claim and still do claim that reverse transcription and the corresponding enzymes are retroviral specific.

A year after Temin discovered reverse transcription in oncoviruses he reported the isolation of a reverse transcriptase from uninfected rat cells.<sup>3</sup> From this he concluded “The finding of a particular ribonuclease-sensitive\* DNA polymerase system [reverse transcriptase] in uninfected cells demonstrates the difficulty of concluding that particulate ribonuclease-sensitive DNA polymerase systems necessarily represent oncogenic viruses”. Thus reverse transcriptase activity is not proof a retrovirus is present. (\*Ribonuclease is an enzyme that degrades RNA molecules. Ribonuclease sensitivity implies the nucleic acid being transcribed is RNA because the enzyme has no effect on DNA and thus its transcription).

In 1972 Temin isolated a reverse transcriptase from “uninfected chicken embryos and cells in culture” and concluded: “it is tempting to assume that this activity is related to normal cell function”.<sup>4</sup> In a lengthy article on retroviruses (which he called RNA tumor viruses or rnaonaviruses) published in 1974 Temin pointed out that reverse transcriptases “with properties similar to rnaonaviruses have been isolated from uninfected cells. The role of cellular endogenous RNA-directed DNA polymerase activity is unclear. However it has been suggested that it may play a role in normal development and carcinogenesis”.<sup>5</sup>

In 1973 Gallo reported a protein in leukemic cells with reverse transcriptase properties “closely related to the enzyme of primate retroviruses...but it must be emphasized that this result does not indicate that the enzyme is specifically found only in leukemic cells...it will be important to determine whether this activity is only found in neoplastic cells or if it is generally present in rapidly proliferating cells”.<sup>6</sup>

In the same year Gallo acknowledged that “Many laboratories subsequently reported the detection of reverse transcriptase in extracts from normal cells.” Gallo and his colleagues themselves reported: “An endogenous and completely RNA-dependent...DNA polymerase [reverse transcriptase] activity was obtained from leukemic blood lymphocytes (and myeloblasts) and from PHA [phytohaemagglutinin, a mitogen derived from kidney beans] stimulated (but not in unstimulated) normal human blood lymphocytes”.<sup>7</sup> Describing the effect of PHA on cells a few years later Gallo wrote “...addition of phytohemagglutinin to these cultures initiates a dramatic transformation in

these cells. They enlarge, synthesise DNA and divide".<sup>8</sup> Note that mitogenic stimulation, most often using PHA, is obligatory to "isolate" HIV; and that the detection of reverse transcriptase activity is often the sole evidence for "HIV isolation".

Several common microbes including bacteria<sup>9</sup> and hepatitis B virus (which is common in AIDS patients and infects T4-lymphocytes<sup>10</sup>) reverse transcribe. In *House of Numbers* Nobel laureate David Baltimore told Brent Leung "There are other forms of reverse transcription that are used in various ways inside the cell...reverse transcription is very widespread".<sup>11</sup> In Australia in 2001 the non-specificity of RT was even publicised in a popular share trading magazine article evaluating the investment potential of biotechnology companies.<sup>12</sup> See [HERE](#) for the current list of retro-transcribing (reverse transcribing) viruses.

Despite ample data to the contrary, in his 1997 interview with the French investigative journalist Djamel Tahj, Montagnier claimed reverse transcriptase activity "is truly specific to retroviruses".<sup>13</sup> When Tahj interviewed Jean-Claude Chermann, the second author of the Montagnier, 1983 *Science* paper<sup>14</sup> he stated: "The second point is related to the detection of RT [reverse transcriptase] activity which is a retrovirus specific enzyme". (DJ Tahj, personal communication). According to a very well-known HIV expert Jaap Goudsmit, reverse transcriptase is "an enzyme absolutely unique to the lentivirus group", that is, only to the group of retroviruses to which HIV is said to belong.<sup>15</sup>

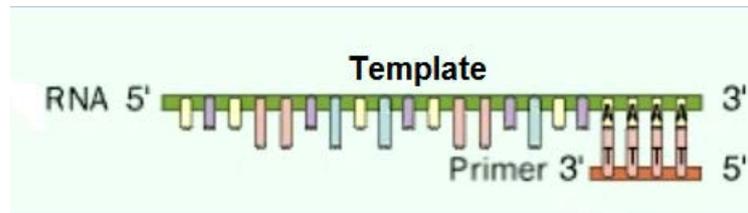
### **Necessary evidence to prove a viral origin of an enzyme**

The discovery of reverse transcriptases in both retroviruses and in non-infected cells led to the question of if and how they could be distinguished from one another. In a paper entitled "Reverse transcriptase of RNA tumour viruses [retroviruses] and animal cells" published in 1976, Gallo stressed the existence of a viral enzyme can be demonstrated only by obtaining the enzyme [protein] from purified virus particles. "For detection and analysis of virus-associated enzyme reactions, it is essential to use virus preparations as free of cellular contaminants as possible [that is, purified]...Generally virus preparations can be concentrated and at least partially freed from cellular contaminants by a combination of differential centrifugation in glycerol and sucrose density gradients. The particles banding at a density of 1.14 - 1.17 gm per ml in a sucrose equilibrium density gradient are generally suitable".<sup>16</sup>

### **Reverse transcription of the synthetic template-primer An.dT<sub>15</sub>**

From the very beginning retrovirologists realised reverse transcription of the "native" retroviral RNA proceeds too slowly to be useful. They found they could increase the rate by substituting a synthetic RNA (ribohomopolymeric) template consisting of the same repeated nucleotide (G,C,A or U). At one end (3') of this RNA is attached a short, synthetic single stranded DNA also formed from a same, repeated nucleotide sequence.<sup>17</sup> The short DNA is called the primer and is required because DNA polymerase, the enzyme that links individual nucleotides to form the DNA polymer, must be "primed" with a small section of DNA in order for the reaction to start.<sup>18</sup> The combination of template and primer is called the template-primer. To measure the reverse

transcriptase activity of a viral enzyme either the purified enzyme or disrupted, purified retroviral particles are added to a mixture of template-primer RNA, the four DNA nucleotides (G, C, A and T) and magnesium or manganese ions. Then the reaction mixture is periodically tested for a DNA product.



There are two ways in which template-primer formulas are written:

1. poly(rX).oligo dY<sub>z</sub>
2. Xn.dY<sub>z</sub> or Xn.dY<sub>-15</sub>

poly = many

rX = the RNA nucleotide; typically in the 100s but usually not stated

oligo = short

dY = the DNA nucleotide

z = the number of dY (often in the range 12-15).

~ = approximately

The DNA primer may be written first. As in (dT)<sub>-15</sub>(A)<sub>n</sub>

When pressed virtually all HIV experts acknowledge the non-specificity of RT activity but claim retroviral RTs can be distinguished from non-retroviral enzymes because the former perform (relatively) better (are more efficiently “utilised” by) using particular template-primers and in the presence of certain cations and at particular pH values.<sup>13, 19</sup> However, as Gallo himself said, the properties of an enzyme cannot be determined without purifying it. No such data were reported by Montagnier and Gallo in 1983/84 or by these scientists or others since. In all HIV research detection of reverse transcription of An.dT<sub>15</sub> is reported from either leukemic cells or cells chemically stimulated by many agents including PHA. Both Gallo and Montagnier accept that the phenomena detected in cultures which are said to prove HIV isolation including reverse transcriptase activity cannot be detected unless cellular division is chemically stimulated using PHA.<sup>20, 21</sup> Cells which divide synthesise DNA using the DNA polymerases β and γ. This means that these cells will, at the same time, reverse transcribe An.dT<sub>15</sub> even if no retrovirus is present in the culture.

In his 1976 paper under the section “Properties that distinguish cellular DNA polymerases from reverse transcriptase” Gallo wrote “As described before, this enzyme [DNA polymerase γ] resembles reverse transcriptase in respect to its high efficiency of transcription of the synthetic homopolymer hybrid primer-template, (dT)<sub>-15</sub>(A)<sub>n</sub>; but polymerase γ is a cellular enzyme probably present in most cells”.<sup>16</sup> He then listed several criteria for distinguishing between viral and cellular reverse transcriptases including the use of antibodies to neutralise DNA polymerase α but in his 1984 isolation papers no

such data were reported. In 1978 Gallo wrote: "...it is now generally agreed that polymerase  $\beta$  can copy (dT)<sub>~15</sub>(A)<sub>n</sub> quite efficiently...DNA polymerase  $\gamma$  and reverse transcriptase have unfortunately been frequently confused because both use (dT)<sub>~15</sub>(A)<sub>n</sub> very efficiently. In addition they have overlapping molecular weights, both are sensitive to sulfhydryl groups inhibitors...possibly polymerase  $\gamma$  can thus be routinely induced under certain conditions and may thus have an important function in all cells".<sup>8</sup> In fact at a 1975 International Conference on Eukaryotic DNA polymerases, DNA polymerase  $\gamma$  was defined as the cellular enzyme that "copies An.dT<sub>15</sub> with high efficiency but does not copy DNA well".<sup>22</sup>

In 1979 Katsuhiko Ono from the Laboratory of Viral Oncology, Hokkaido University, Kita-Ku, Sapporo, Japan wrote "Among a number of template • primers, (rA)<sub>n</sub>•(dT)<sub>12\_18</sub> has been most frequently employed since RT shows high activity with this template•primer. However, the problem is that the cellular DNA polymerases (pol  $\beta$  and poly  $\gamma$ ) also effectively utilize the same template • primer".<sup>23</sup> In other words before the AIDS era both Gallo and Montagnier knew that reverse transcriptase activity can be detected in cell cultures using the template-primer An.dT<sub>15</sub> even in the absence of both viral and cellular reverse transcriptases.

A 2004 review of DNA polymerase  $\gamma$  states DNA polymerase "is the only cellular DNA polymerase that can utilize effectively a ribohomopolymeric [RNA] template...The DNA polymerase activity of pol  $\gamma$  [DNA polymerase  $\gamma$ ] has been assayed...on poly(rA) : oligo(dT)...Pol exhibits a broad pH optimum (7.5–9.5), absolutely requires a divalent cation (Mg<sup>2+</sup> or Mn<sup>2+</sup>, depending on the template primer used), and has a high affinity for dNTPs [deoxy nucleotide triphosphates]...Although pol  $\gamma$  was thought not to utilize natural sequence RNA as a template, a recent study shows that it does so with a slightly higher catalytic rate than HIV-1 reverse transcriptase". Furthermore, DNA polymerase  $\gamma$  "is unique among the cellular DNA polymerases with regard to its strong inhibition by anti-HIV-1 reverse transcriptase drugs".<sup>24</sup> Thus, the reverse transcription of the template-primer An.dT<sub>15</sub>, cannot be considered synonymous with the detection of a retroviral reverse transcriptase, much less a specific retrovirus.

The reason HIV experts interpret reverse transcriptase activity as a retroviral infection is their failure to use controls. A control is an "Essential part of a scientifically valid experiment, designed to show that the factor being tested is actually responsible for the effect observed. In the control experiment all factors, apart from the one under test, are exactly the same as in the test experiments, and all the same measurements are carried out". In experimental biological research it is accepted that experiments must include:

1. The choice and use of appropriate controls.
2. The experiments are done blindly.

The reason for controls is to ensure the phenomena detected are not the result of unknown or unforeseen non-viral factors. Controls involve testing cultures taken from sick individuals not at risk of developing or having the

disease being studied. Furthermore, since patients belonging to the AIDS risk groups are exposed to oxidising agents and their tissues are known to be oxidised,<sup>25-28</sup> control patients must have laboratory abnormalities similar to AIDS patients, including hypergammaglobulinaemia, exposure to oxidising agents, for example, chemotherapy, radiation, or the immunosuppressive agents used in organ transplantation. Before the AIDS era Gallo, Weiss and other scientists published papers showing that “The expression of endogenous retroviruses can affect the results of seemingly unrelated experiments”,<sup>29, 30</sup> that is, experiments where a retroviral infection is not present. And endogenous retroviral genomes are present “in all of us”.<sup>31</sup> This means that without suitable controls scientists may wrongly infer a retrovirus infection.

Since researchers can be biased consciously or subconsciously, for the sake of scientific accuracy the experiments must be done blindly. This means the scientist who analyses the results must not know the identity of test and control experiments until all analyses are complete.

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## Addenda

Montagnier's use of poly(A) · (dT)<sub>12-18</sub> as template-primer in the first isolation of HIV.<sup>14</sup>

All HIV experts use this template-primer.

Fig. 1. Analysis of virus from patient 1 on sucrose gradients. Cord blood T lymphocytes infected with virus from patient 1 were labeled for 18 hours with [<sup>3</sup>H]uridine (28 Ci/mole, Amersham; 20 μCi/ml). Cell-free supernatant was ultracentrifuged for 1 hour at 50,000 rev/min. The pellet was resuspended in 200 μl of NTE buffer (10 mM tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and was centrifuged over a 3-ml linear sucrose gradient (10 to 60 percent) at 55,000 rev/min for 90 minutes in an IEC type SB 498 rotor. Fractions (200 μl) were collected, and 30 μl samples of each fraction were assayed for DNA polymerase activity with 5 mM Mg<sup>2+</sup> and poly(A) · oligo-(dT)<sub>12-18</sub> as template primer; a 20-μl portion of each fraction was precipitated with 10 percent trichloroacetic acid and then filtered on a 0.45-μm Millipore filter. The <sup>3</sup>H-labeled acid precipitable material was measured in a Packard β counter.

## Properties of reverse transcribing enzymes

TABLE III  
DISTINGUISHING MOLECULAR WEIGHTS AND PRIMER-TEMPLATE PROPERTIES OF CELLULAR AND RETROVIRUS DNA POLYMERASES

Property	Cellular DNA polymerases			Retrovirus DNA polymerase	References
	α	β	γ		
Molecular weight	110 000– 220 000	45 000	110 000– 330 000	avian: 160 000 mammalian: 70 000	37,97,98,251,503
Preferred primer-template	"activated" DNA	(dT) <sub>≈15</sub> · (dA) <sub>n</sub>	(dT) <sub>≈15</sub> · (A) <sub>n</sub>	avian: (dG) <sub>≈15</sub> · (C) <sub>n</sub> mammalian: (dT) <sub>≈15</sub> · (A) <sub>n</sub>	37,50,70,96,504
Ratio of activities: $\frac{(dT)_{\approx 15} \cdot (dA)_n}{(dT)_{\approx 15} \cdot (A)_n}$	>>1	>1	<0.1	<0.01	37,70,90,91,98
Sensitivity to MalNEt	very sensitive	resistant *	moderately sensitive	moderately sensitive	46,70,235,505
Ability to copy natural RNA template	No	No	No	Yes	69,96,97,98,506
Ability to copy poly (2'-O-methylcytidylate)	No	No	No	Yes	70,290,291
Ability to use synthetic RNA-primed DNA template	Yes	Yes	Yes	No	65,70,71,411

Sarngadharan MG, Robert-Guroff M, Gallo RC. DNA polymerases of normal and neoplastic mammalian cells. *Biochim Biophys Acta* 1978;516:419-87.

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