

## ANALYTICAL REVIEW

### **RNA-Dependent DNA Polymerase in Viruses and Cells: Views on the Current State**

By ROBERT C. GALLO

**B**Y NOW the fact that some enzymes that synthesize DNA (DNA polymerases) can use RNA templates (RNA-dependent) is well established. The story, of course, began with the efforts of Howard Temin and some other tumor virologists to explain how an RNA tumor virus could produce a stable genetic trait, a characteristic passing from cell to daughter cell, namely, neoplasia, without the virus itself carrying DNA. The idea, proposed in 1964 by Temin to explain this phenomenon, involved the synthesis of a DNA intermediary using the viral RNA as template. Temin further proposed that this DNA would then be integrated into the host genome and account for neoplastic transformation. Furthermore, the integrated DNA would also be involved in synthesis of new virus RNA. This idea of replication of RNA tumor viruses through a DNA intermediate, not through an RNA intermediate (as do other RNA viruses), is the basis of the provirus hypothesis. The support for these concepts at the time was based on indirect information; for example, Temin,<sup>1</sup> Bader,<sup>2</sup> and others<sup>3</sup> had shown that actinomycin D inhibited replication of Rous sarcoma virus (RSV). Since at the concentrations used actinomycin D specifically inhibits DNA-templated reactions, and RSV is an RNA virus, the speculation was made that some stage of viral replication involved a reaction using DNA as template. The observations that infection of cells by RSV requires synthesis of DNA different from that synthesized in S phase of the cell cycle<sup>4</sup> and that cells transformed by RNA tumor viruses contain new DNA, which hybridizes with viral RNA,<sup>5</sup> lent support to this proposal. However, the concept did not gain widespread approval because (1) much of the evidence, as Temin realized, was indirect, initially based for the most part on inhibitor

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Abbreviations used in text: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelocytic leukemia; RDDP, RNA-dependent DNA polymerase ("Reverse Transcriptase"); DDDP, DNA-dependent DNA polymerase; RLV, Rauscher leukemia virus; MSV, murine sarcoma virus; RSV, Rous sarcoma virus; AMV, avian myeloblastosis virus; rA.dT, the synthetic RNA-DNA hybrid consisting of homopolymers or oligomers of riboadenylate and deoxythymidylate; rA.rU, the synthetic RNA duplex consisting of homopolymers of riboadenylate and ribouridylate; dC.dG, the synthetic DNA duplex consisting of homopolymers of deoxycytidylate and deoxyguanylate; d(AT), the synthetic DNA consisting of an alternating copolymer of dA and dT.

experiments, and (2) what is perhaps more important, the concept was new and not in the traditional scheme of bacterial molecular biology.

However, as it is now known to almost every biologist, in June 1970 Temin and Satoshi Mizutani<sup>6</sup> and David Baltimore<sup>7</sup> independently published their findings of an RNA-dependent DNA polymerase (RDDP) in RSV and in Rauscher leukemia virus (RLV). These findings were quickly confirmed and extended in several laboratories, especially those of Spiegelman<sup>8,9</sup> and Green.<sup>10</sup>

The current concept of this process involves at least two steps, as shown below:



The precise mechanisms are still very debatable (see below), but the end result, synthesis of DNA from the virus 70S RNA, can be considered as established. It appears from the elegant work of Duesberg et al. that the entire 70S RNA is transcribed into DNA.<sup>11</sup>

Both RNA-dependent and DNA-dependent DNA polymerase activities have been detected. These activities have not as yet been separated. Both are probably from one protein, and there is no evidence to indicate that the activities arise from more than one catalytic site, and suggestive evidence indicates that the sites are the same, e.g., when partially purified RLV polymerase is saturated with RNA template there is no increase in the activity when DNA is added (Reitz, M., Sarin, P., and Gallo, R., unpublished results).

One of the most effective templates for the virion polymerase is an RNA-DNA hybrid (see below).<sup>9</sup> This is to be expected since a hybrid structure is the product of the first step of the proposed endogenous reaction. ("Endogenous reaction" here refers to DNA synthesis from the viral RNA.) After synthesis of double-stranded DNA from the hybrid, the DNA is thought to be integrated into the host cell's genome. Recently, Temin and his colleagues<sup>12</sup> reported that RSV also contained a DNase (endonuclease) and also provided evidence for a DNA ligase and exonuclease.<sup>13</sup> If these latter activities are, in fact, part of the virus internal "machinery," then all the events required for the synthesis of double-stranded DNA and its integration into the cell genome can in principle be accomplished.

#### DISTRIBUTION OF "REVERSE TRANSCRIPTASE" IN RNA VIRUSES

The total number of RNA virus systems now examined for the presence of RDDP is at least 40. Thirty-three of the 40 were positive, and of these, 27 are known oncogenic viruses. Of the remaining six, four are *probably* oncogenic and, until very recently, two (the Foamy virus and Visna virus) were thought not to have oncogenic potential (Table 1). The Visna virus is the causative agent of a chronic neurological disease in sheep. However, if the idea initiated by Temin is valid for neoplasia, it might also be valid for other disorders induced by RNA viruses that also involve stable genetic alterations. Indeed, Schlom et al.<sup>14</sup> have suggested an investigation of tissues of patients with multiple sclerosis for presence of this type of enzyme activity. In any event, very recent information indicates that even the Visna virus is capable of producing

Table 1.—Summary of RNA Viruses Containing DNA Polymerase

Virus	Source	Activity
<b>Viruses of Known Oncogenic Potential</b>		
Rous sarcoma		
(RAV-1)	CEF*	+
(Prague)	Cell culture (chicken)	+
(Schmidt-Ruppon, B-77)	Cell culture (chicken)	+
Avian leukosis (Mc 29)	CEF	+
Avian reticuloendotheliosis		
(Twiehaus agent)	CEF	+
Avian myeloblastosis	Chicken plasma	+
	Cultured myeloblasts	+
<b>Murine leukemia</b>		
Rauscher	Plasma	+
Rauscher	MEF †	+
Rauscher	JLS-V5 cell line	+
AKR	Cell culture (rat)	+
Moloney	JLS-V9 cell line	+
<b>Murine sarcoma-leukemia complex</b>		
Moloney	Mouse tumors	+
Moloney	MEF	+
Moloney	78A1 rat cells	+
Harvey	MEH mouse cells	+
Kirsten	NRK cells	+
Murine mammary tumor	Paris R III milk	+
	C <sub>3</sub> H milk	+
<b>Feline leukemia</b>		
Ricard	Cell culture (feline)	+
Thielen	Cell culture (feline)	+
Gardner	Cell culture (canine)	+
<b>Feline sarcoma</b>		
Ricard	Cell culture (marmoset)	+
Gardner	Cell culture (feline)	+
Gardner	Cell culture (canine)	+
Hamster sarcoma	B34 hamster cells	+
Hamster leukemia virus	LSH hamster cells	+
<b>C-Type and/or B-Type Viruses of Unproven Oncogenicity</b>		
Mammary tumor	R35 rat cells	+
Mammary tumor (Mason)	Cell culture (monkey)	+
Viper	VSW cells	+
Visna	Choroid plexus cells (sheep)	+
Mammary tumor (human)	Human milk	+
<b>Nononcogenic Viruses</b>		
Newcastle disease	Allantoic fluid	—
Influenza (A and WSN)	Allantoic fluid	—
Reo	L-cells	—
Vesicular stomatitis	BHK cells	—
Polio	HeLa cells	—
Sendai	Allantoic fluid	—
Respiratory syncytial	HEP-2 cells	—
Simian foamy virus	Monkey kidney	+

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\* CEF, chick embryo fibroblasts; † MEF, mouse embryo fibroblasts.

neoplastic transformation of normal mouse cells (Takemoto, K., and Stone, L. B., *J. Virol.* In press) so that the *only presently known exception of the presence of virion reverse transcriptase activity and oncogenic potential is the Foamy virus.*

#### RDDP IN CANDIDATES FOR HUMAN TUMOR "VIRUSES"

Three recent findings of RDDP activity in particles obtained from humans illustrate the potential for major usefulness of this enzyme assay in helping to determine whether a particle is, in fact, a virus and adds support to the possibility that the particle may play a role in the etiology of the virus-associated disease.

##### *Human Milk "B Particle"*

D. Moore and his colleagues<sup>15</sup> have shown that milk from patients with breast cancer contains particles morphologically similar to the B particles that produce mouse mammary tumors. A recent report<sup>16</sup> indicates that these particles contain RDDP activity. Although additional analysis of the product of the reaction is needed before definitive conclusions can be drawn, this report certainly supports the notion that these particles are viruses and obviously adds credence to the concept that they may be oncogenic.

##### *Australian Antigen*

S. Hirschman (personal communication and reference 17) has detected RDDP in a particulate fraction of plasma (obtained after high-speed centrifugation) of patients with Australian antigen-positive hepatitis. Detailed analyses of the product has not yet been completed, but the reaction apparently was shown to be dependent on a natural RNA, RNA endogenous to the particulate fraction. Examination of the particulate fractions from normal sera were negative for RNA-dependent DNA polymerase. Lack of any polymerase activity in normal sera has been reported by Kiessling et al.<sup>18</sup> but has not been our experience.<sup>19</sup> (DNA-dependent DNA polymerase is detectable in some normal sera). If this antigen does indeed contain RDDP is this particle an RNA tumor virus? Confirmation of this finding is obviously of great importance.

##### *"Human" Cell C Particle*

The M. D. Anderson tumor virology group has just published their findings of a type C virus in cells obtained from a pleural effusion of a patient with American-Burkitt type lymphoma.<sup>20</sup> The particles, which are now being successfully produced from a cell line (ESP-1) established from the original source, do not cross-react with specific antigens of rat, hamster, or cat-type C-particles. However, there is some recent indication of cross-reactivity with Gs-1 antigen of mouse leukemia virus (Gilden, R.: personal communication). Karyotype and immunologic studies indicate that the cell line is indeed human. In a collaborative study with the Texas group, we have found RDDP activity utilizing particles purified by equilibrium gradient centrifugation. We have shown a polymerase is present which catalyzes out an endogenous, RNase-sensitive, detergent-dependent DNA synthesis that is restricted to the 1.15–1.16

density region of a sucrose gradient (density typical of intact C-particles). The same enzyme readily utilizes nucleic acid from the feline leukemia virus.<sup>21</sup> It will obviously be important now to determine, among other things (1) whether antibodies can be found in sera from other patients with lymphoma that inhibit this polymerase, thereby suggesting a role of similar particles in these patients; (2) whether the DNA made by the human C-particle will hybridize with cellular RNA from other human cancer patients; and (3) to determine with certainty whether the *virus* is indeed human.

#### PARTICULATE FRACTION FROM LEUKEMIC SERUM

We have found that particulate fractions from sera of several leukemic patients (ALL, AML, and CML) contain DNA polymerase activity.<sup>22</sup> However, in *no case* was endogenous, RNase-sensitive detergent-dependent DNA synthesis observed. All activity was either dependent on the addition of DNA, RNA-DNA hybrids, or if endogenous activity was found it was destroyed by preincubation with DNase but not RNase. Activity was particularly noted in patients with high white cell counts. We interpret these findings to mean that the polymerase activity was from DNA-dependent DNA polymerase liberated from dead or dying cells, probably associated with plasma membranes or other cellular fragments and *not* from an RNA tumor virus. Kiessling et al.<sup>18</sup> similarly reported DNA polymerase activity in sera of two patients with CLL. This preparation showed activity with exogenous DNA templates. Of interest, when the polymerase activity of the particulate fraction was analyzed by isopycnic centrifugation the activity banded in the density region where the RNA tumor virus core particles are found; however, no endogenous RNase-sensitive activity was reported.

#### PRACTICAL ASPECTS OF ASSAYS FOR RDDP IN VIRUSES

In assays for viral polymerase activities, care must be taken that the virus preparation is free of cellular contaminants, and the difficulties of achieving this are well known. Not only are cellular fragments common contaminants, but apparently even enzymes may adhere to the outer envelope. However, it has been shown by Gerwin et al.<sup>23</sup> that the polymerase of tumor viruses is associated with the "nucleoid" of the virus, not with outer components. The preparation of the viral polymerase usually involves treatment with a nonionic detergent to "solubilize" the enzyme. This generally increases enzyme activity markedly, but the acceptable concentration range is narrow. High concentrations of detergent inhibit the activity. These agents allow for "solubilization" of the enzyme from viral structural components.

The assays either measure an *endogenous* templated reaction (no RNA or DNA added) utilizing the virus 70S RNA or an *exogenous* templated reaction utilizing added DNA, RNA, or RNA-DNA hybrids. Synthetic hybrid templates were introduced by Spiegelman<sup>9</sup> and Temin<sup>12</sup> and their associates. They observed that some double-stranded synthetic homopolymers containing one polydeoxyribonucleotide strand and one polyribonucleotide (e.g., rA.dT) were far superior templates for the RDDP than the endogenous RNA. These templates do provide ancillary information about the nature of the enzyme, and

since the virus RDDP has great affinity for them, they can be very sensitive indicators of this activity. In fact, they have been advocated and used by Spiegelman<sup>24</sup> for preliminary searches for this or related enzyme activity in cells, and have now been used in several other laboratories including our own.

It is now quite clear that although the virus RDDP does have relatively great affinity for these synthetic templates, they are far from specific. In a comparison of the template preferences of *Escherichia coli* DNA polymerase I and the DNA polymerase of AMV, Baltimore<sup>25</sup> showed that although the *E. coli* DNA-dependent enzyme will "read" off the ribohomopolymer strand, it has marked preference for the deoxyribohomopolymer strand. Conversely, the AMV polymerase will not use any deoxyribohomopolymer with the exception of poly dC. In our hands, several partially purified DNA polymerases have been examined (from normal or neoplastic proliferating cells) and most will use the RNA strand of rA.dT. Therefore, though useful in screening procedures, activity with these templates is not specific and is certainly not proof of a virus RDDP or for that matter of any RDDP.

To establish the presence of a "reverse transcriptase" in any virus it would seem that a minimum initial requirement would include: (1) demonstration of an endogenous reaction that is RNase sensitive, i.e., an RNA-dependent reaction; (2) requirements for all four deoxynucleoside triphosphates (to indicate polymer formation and not just terminal addition of a deoxynucleotide); (3) proof that the product is DNA; (4) polymerase activity that bands in the density characteristic of virus that depends on detergent treatment. It would obviously also be desirable to (5) show complementarity between the RNA template and the DNA product and (6) to demonstrate that one of the two strands is RNA. Even with the first three criteria established it is possible that some virus preparations contain small amounts of DNA<sup>26</sup> thereby enhancing a DNA-dependent DNA polymerase reaction. The last two objectives have not been established for every RNA virus reported to contain RDDP activity; for example, it has not yet been established for the particles from human sources, but one can be certain that these are in progress.

#### BIOLOGICAL FUNCTION OF VIRUS RDDP

The very presence of DNA polymerase in RNA tumor viruses, of course, suggests a role for the enzyme in initiation of neoplastic transformation. In support of this, Hanafusa<sup>27</sup> has shown that a mutant of RSV termed RSV $\alpha$  (0), which has no infectious capacity, is lacking in polymerase activity. However, after many years of investigations on the *E. coli* DNA polymerase I, its biological function in the whole bacterium remains uncertain. More information will have to be obtained from studies with mutants, as well as perhaps the use of selective inhibitors, before we can be certain of the role of this enzyme activity in tumor formation or in cell growth.

#### PRESENCE OF RDDP IN CELLS

##### *Neoplastic Cells*

After the reports of RDDP in RNA oncogenic viruses, it was of interest to examine cells, and in particular, human leukemic cells, for the presence of

this type of enzyme. First, for the obvious reason that it might be a sensitive index of the presence of an RNA tumor virus or virus product, thereby implicating a role of RNA tumor viruses in the pathogenesis of the disease. Second, even if RDDP is present but is not the same as the tumor virus enzyme, the presence of an analogous polymerase in leukemic cells might account for differences in RNA species between normal and leukemic cells. These differences might be involved in the faulty maturation of the latter,<sup>28-33</sup> or an altered or more abundant activity in cells might produce neoplastic transformation as suggested in the provirus hypothesis of Temin (see below). Third, it was possible that this was general activity of all DNA polymerases, not described previously simply because it had not been looked for under appropriate experimental conditions. Fourth, it was time to begin a systematic comparative investigation of the properties of the DNA polymerase(s) of human normal and leukemic leukocytes. These enzymes, whether virus specific, host specific (purely host originated), or a combination of both are likely to be the key proteins involved in regulation of growth and have not previously been purified or well characterized in these cellular systems.

Last year we reported detecting RNA-dependent DNA polymerase activity in partially purified extracts prepared from lymphoblasts of three patients with very high count (greater than 100,000/cu mm) untreated ALL. The activity was inhibited by the rifampicin derivative, N-demethylrifampicin, but *not* by rifampicin itself.<sup>31,32</sup> At that time a similar activity was not found in phytohemagglutinin (PHA)-stimulated normal lymphocytes. We feel that the best normal cell comparisons for leukemic cells are normal peripheral blood lymphocytes for CLL and PHA-stimulated lymphocytes for ALL.<sup>34,35</sup> (For AML there is no available comparable control.) Although there is no perfect normal control for human leukemic cells, we feel that in the above systems the control and leukemic cells are at least comparable in morphology and metabolic activity. We have also emphasized<sup>32</sup> that lack of activity in normal lymphocytes might have been due to limits of sensitivity of the assay and that other normal cells might contain this activity. Temin,<sup>35</sup> Baltimore,<sup>36</sup> and later our laboratory<sup>31,32</sup> suggested that this activity might play a role in cytodifferentiation. Finally, we have stressed<sup>31,32,35</sup> that the mere presence of RDDP activity does not indicate the nature of its source. Its presence, therefore, does not prove the existence of an RNA tumor virus. There are, however, similarities between the RDDP of leukemic cells and those obtained from RNA tumor viruses. We have been able to show that from leukemic cells an activity is present that is dependent on exogenous RNA. RNase but not DNase destroys the template. The product synthesized is DNA as shown by sensitivity to DNase but not RNase. The reaction requires all four deoxynucleoside triphosphates, and initially we showed that the enzyme used both natural templates as well as synthetic double-stranded RNA. Confirmation of these results with human leukemic cell extracts and using natural templates has now been achieved in at least three other laboratories.<sup>36-38</sup> In addition, evidence for the presence of RDDP in infected neoplastic cells has been reported by Temin.<sup>39</sup> The approach used was to show that crude extracts of these cells (but not normal cells<sup>39</sup>) showed endogenous DNA synthesis that was destroyed by treatment of the crude cell-

ular extract with RNase prior to the initiation of polymerase assay. Spiegelman and associates<sup>40</sup> examined a wide range of biological systems utilizing synthetic double-stranded polydeoxyribonucleotides, the homopolymers dC.dG, and the DNA hybrid rA.dT, and apparently have consistently found much higher rA.dT/dC.dG ratios with neoplastic cells than with normal cells, with the exception of tissue culture cells and early embryos (see below). Virtually all leukemic tissue culture cells and all solid tumors, except acute monocytic leukemia (Spiegelman, S., personal communication), showed this relatively high activity with rA.dT.

Our subsequent investigations have remained primarily with human leukocytes. We have started with a large quantity of cells and have attempted partial purification of this activity in each case. We have sought activity with natural templates as well as synthetic templates. With the use of rA.dT our results are essentially in agreement with those of Spiegelman and his colleagues. With natural RNA templates, e.g., phage MS2, reovirus RNA, or mammalian 28S RNA, our positive findings are much more restricted. However, it is premature to state what percentage are positive, since it is not certain whether natural templates are simply just too insensitive (and low activities are missed) or whether the reactions with these templates are carried out by an enzyme distinct from the one that carried out the rA.dT directed reaction. So far whenever we have obtained fractions having activity with natural templates, the same fraction has always showed activity with rA.dT.

### *Normal Cells*

When the sensitivity of rA.dT for detection of the virion RDDP became known, several laboratories utilized this template to find *preliminary* data that might suggest the presence of RDDP, especially after partial purification. For example, Spiegelman et al.<sup>40</sup> stated that his laboratory has found abundant activity in embryos at early stages as well as neoplastic cells. Similarly, we noted activity with rA.dT in normal human lymphoblasts.<sup>34,35</sup> However, these activities were not assumed to be RDDP since the lack of specificity with this template was soon apparent. Scolnick et al. noted activity with rA.dT and rA.rU with the established nontransformed mouse cell line (BALB/3T3) and with human fibroblasts in culture and concluded RDDP activity was present in normal cells in tissue culture.<sup>41</sup> With the exception of a recent preliminary report by Penner et al.,<sup>38</sup> there are no published data to indicate the existence in normal cells of *RDDP capable of using natural templates*, and in this one report template instruction by *single-stranded RNA* was not shown and it would not be determined from the data presented whether this was a terminal addition enzyme or a true polymerase. These authors state they have confirmed our findings of RDDP in ALL and extended these positive findings to CLL. If the findings in normal lymphocytes are verified, they will have strong implications to theories of information transfer with RNA for antibody formation. We have recently found considerably more activity from PHA-stimulated lymphocytes than we previously reported, using more purified enzyme preparations. This new activity, although stimulated by some natural RNA templates, double-stranded but so far *not* with any *single-stranded RNA*.



## IS THE ENZYME IN SOME NEOPLASTIC CELLS THE SAME AS THE VIRUS ENZYME?

The answer to this question is not settled. The criteria for identity are not easy to establish. It is easy to find certain points of dissimilarity. For example, (1) the polymerase from RLV elutes from a phosphocellulose column at a salt concentration slightly different from the enzyme of leukemic cells. However, this approach may not be meaningful until enzymes from both virus and cells are completely purified, since variable and different contamination with other factors (cell or virus) might account for these differences. (2) Furthermore, as emphasized previously, the fine biochemical properties of a human RNA tumor virus may differ from those of animal origin just as some of the properties of the RDDP from mammalian C-type virus appear to differ from avian. The recent report of Schlom et al.<sup>16</sup> on RDDP activity from virus-like particles isolated from human milk suggests that this may be the case. They found that the activity in these particles was only enhanced about two-fold by the hybrid rA.dT in comparison to the endogenous RNA-directed reaction. This is considerably less than that found with AMV or RLV polymerase.<sup>9</sup> (3) No one has shown that any "cellular" enzyme from tumor cells can use 70S RNA from RNA tumor virus as template. However, not until very recently<sup>42</sup> did anyone report success with this even with the RDDP from viruses, i.e., to solubilize the polymerase, completely separate it from its endogenous RNA, and then obtain DNA synthesis with addition of the tumor virus 70S RNA to the reaction mixture. Further, some specificity was shown, i.e., the RDDP from RSV prefers its own 70S RNA rather than RNAs from other sources.<sup>42</sup> With human tumor cells we are obviously handicapped by not previously having 70S RNA from a proven human RNA tumor virus. Moreover, the cellular enzyme preparations are, of course, more complex, requiring greater purification efforts and consequently greater chances for loss of activity. We have found difficulty in removing all traces of nucleases from partially purified cellular polymerases while still retaining polymerase activity. Since the single-stranded virus 70S RNA is extraordinarily susceptible to RNase destruction, these nucleases must be removed before we can be certain that a cellular RDDP lacks template activity with 70S RNA. As stated above, certain cellular (leukemic) DNA polymerases appear to be capable of utilizing a single-stranded RNA as template (Sarin, P., Wasserman, P., and Gallo, R., in preparation). The possibility that a contaminating oligodeoxynucleotide is present and necessary for the reaction to initiate has not been ruled out. However, the reaction is RNase sensitive, indicating that if the DNA fragment is present it serves as a primer while the RNA acts as the template, as shown below.



The reaction may then proceed building off the 3'-OH group of the DNA fragment primer that is hybridized to the RNA, while the RNA acts as the template. On the other hand, according to Spiegelman (personal communica-

tions) the RDDP of RNA tumor viruses can work with single-stranded RNA purified in a manner that should remove any DNA fragment hybridized to RNA. This, of course, would have to be done with a highly purified viral enzyme to be sure the enzyme preparation does not supply a contaminating DNA fragment. If this is the case and cellular RDDP does require a DNA primer, the enzymes will obviously be mechanistically distinct. However, cellular RDDP still will have implications for reversal of information flow and remain a critical component of Temin's protovirus theory (see below).

There are, of course, some striking similarities between virion RDDP and the cellular enzyme. Both human leukemic and viral RDDP: (1) can utilize double-stranded synthetic ribohomopolymers but both prefer RNA-DNA hybrids such as poly dT.rA; (2) work very well with poly d(AT); (3) elute from gel filtration columns in the same volume,<sup>41</sup> suggesting similar molecular sizes; and (4) are inhibited to a similar extent by certain rifampicin derivatives,<sup>31,32,41,43</sup> which has minimal effects on some DNA-dependent DNA polymerases and are not inhibited by rifampicin itself.<sup>31,32,43</sup> Green and his associates<sup>43</sup> have argued that since certain rifampicin derivatives inhibit the polymerase of three different RNA tumor viruses (MSV, FeLV, and AMV) these three different species of RNA tumor viruses must possess a common structural feature. The RDDP from cells is inhibited to the same degree by the same derivatives (Yang, S., Herrera, F., Smith, G., and Gallo, R., in preparation; Todaro, G., personal communication). Of course the argument depends on proof that these rifampicin derivatives act only by complexing with the enzyme and not by interacting with the nucleic acid templates, which has not been adequately proven. Apparently, within certain concentration ranges some rifampicin derivatives inhibit only the RDDP from RNA tumor viruses and not other DNA polymerases, e.g., *E. coli*.<sup>43,44</sup> If these data are extended, i.e., to include a number of other DNA polymerases, then it would be additional evidence for similarity between cellular and viral enzymes, since as stated above the derivatives that appear to be potent inhibitors of the virus RDDP also inhibit the enzyme from leukemic cells. Similarity between the viral and cellular enzymes, by comparison of the efficacy of several templates, inhibitor effects, and mobility on columns, has been stressed by Scolnick et al.<sup>41</sup>

#### ARE DNA POLYMERASES DESCRIBED IN NORMAL CELLS IDENTICAL TO THOSE OF LEUKEMIC CELLS?

In relatively crude extracts differences in template preferences for leukemic and normal cells have been noted.<sup>40</sup> On the other hand, with tissue culture cells derived from normal donors some similarities have been described.<sup>41</sup> It can be said with certainty that at least normal proliferating cells do contain an enzyme activity(s) that will utilize the ribohomopolymer strand of dT.rA,<sup>35,40,41</sup> but whether this activity is identical to RDDP of leukemic cells has not been established and must await further purification.

We are presently comparing the template specificities, effects, of inhibitors, and physical properties of each DNA polymerase isolated and partially purified from RLV, normal lymphocytes (fresh), "normal" lymphoblasts (tissue culture established), leukemic lymphoblasts, and *E. coli*. As noted above with the leu-

kemic (ALL) RDDP, we recently succeeded in obtaining DNA synthesis with a single-stranded RNA, 28S human rRNA. We do not yet know if a true copy of the RNA is synthesized, but so far this reaction has not been found with the polymerases from normal lymphocytes.

Another and important approach will be to test for cross-reactivity of antibodies prepared against these purified polymerases. Aaronson et al.<sup>45</sup> (Todaro, G., personal communication) have very recently found that antibodies directed against RDDP of RLV not only inhibit RLV RDDP, but also inhibit the RDDP of other murine RNA tumor viruses, feline leukemia virus, and feline sarcoma virus, but do not inhibit RDDP from AMV, Visna virus, Foamy virus, and mouse mammary tumor virus.

A question that is now of immediate great interest is whether the antibodies against the animal tumor virus RDDP will inhibit RDDP from human cells. These experiments are now in progress. The negative cross-reactions with RDDP of some oncogenic viruses indicate that positive findings with human RDDP may not be found. What are needed are antibodies prepared against a *human* tumor virus RDDP, and this may be possible now with the type B particle isolated by Moore and his colleagues<sup>15,16</sup> and the C-type virus of Priori et al.<sup>20,21</sup>

#### POSSIBLE CLINICAL APPLICATIONS AND APPROACHES

##### *Usefulness as "Footprint" of RNA Tumor Virus*

As described above, the assays for presence of RDDP may not be specific enough to distinguish nonviral cellular DNA polymerases from the RDDP of RNA tumor viruses. If the DNA polymerases of normal cells do not show activity with tumor virus 70S RNA and the leukemic cells RDDP does then this *might* be a criterion for viral origin of the enzyme. Another approach is to find a particulate fraction, in tumor but not in normal cells, that contains an endogenous RNAase sensitive, DNA polymerase activity which is activated by nonionic detergents.

The discovery of RDDP has opened up other approaches in the search for "footprints" of RNA tumor viruses other than just the assay of this activity. For example, if RDDP is present in sufficient quantity in transformed cells, RNA-DNA hybrids may be detected. We are looking for these products in cell extracts and in plasma by biochemical and immunologic approaches. The latter may be possible through the use of antibodies present in the sera of patients with systemic lupus erythematosus directed against these hybrid structures (Talal N., and Gallo, R., in preparation). A second approach will be to use RDDP from a human RNA tumor virus to make DNA and then to use this DNA in molecular hybridization experiments to look for complimentary RNA in various human tumors. This may be done in the very near future in view of the possibilities that the C-type virus<sup>20</sup> of Priori et al. and the viruslike particles from human milk isolated by Moore and his associates<sup>15</sup> may represent human RNA tumor viruses. Finally, antibodies may be prepared against human virus RDDP. The antibodies could then be used in cross-reactivity studies with human tumor cells by determining if it inhibits a specific polymerase isolated from these cells.

### *Diagnostic and Prognostic Use*

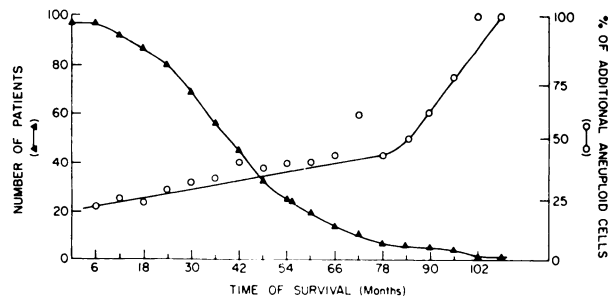
The observation made by Spiegelman and his associates that the ratio of DNA polymerase activity with a DNA hybrid (rA.dT) to DNA (dC.dG) was invariably much higher in crude extracts of leukemic cells compared with normal leukocytes, even proliferating normal leukocytes, and that the rA.dT activity rapidly diminished following chemotherapy,<sup>24,40</sup> naturally led to the interesting proposal that these assays might have diagnostic and prognostic utility.<sup>24</sup> This biochemical approach (as well as some other, e.g., lower nuclease activity and higher thymidine kinase in leukemic blasts compared to mature cells) might be a more sensitive index than morphology, and it will be of interest to see if this approach is clinically useful. By comparing these assay results with the standard hematologic morphologic examinations, it will not be difficult for clinical laboratories to rapidly accumulate enough information to determine if, in fact, this approach is useful and worth the effort.

### *Therapeutic Approaches*

**General Concepts:** The rationale in our laboratory for the investigation of DNA polymerase inhibitors is based on several factors. (1) Selective inhibitors may distinguish one DNA polymerase from another, and if qualitative or even quantitative differences exist between DNA polymerases of normal and neoplastic cells, selective chemotherapy may be possible, whether or not the polymerase of neoplastic cells is from a virus. (2) A selective inhibitor of RDDP will be useful in determining if this activity is necessary for neoplastic transformation and/or maintenance of the neoplasia. (3) A selective inhibitor of RDDP may be clinically important, e.g., if RDDP is necessary for *maintenance* of the transformed state, then inhibition may result in selective destruction of these cells. A fourth consideration is discussed in detail below.

**Is "Reinfection" Responsible for Some Relapses in Leukemia?** Theoretically, if the pathogenesis of human leukemia involves an RNA virus and if RDDP in a human leukemic cell is viral in origin, then inhibition of this activity may have no effect on a cell already transformed. That is, expression of viral genome activity (including RDDP) may not be necessary for maintenance of the neoplastic state. However, many patients with acute leukemia are readily put into remission, but relapse occurs resulting in reappearance of leukemic blast cells. Does relapse occur solely because of a failure to kill the last neoplastic cells with chemotherapeutic agents? This widely held concept may be true in most instances, but I doubt that this is the full explanation. We now see evidence that some patients survive years without any signs of their disease, and then sometimes suddenly leukemic blasts are found in abundance. It is reasonable to believe that in these patients the inciting agent may still be present resulting in subsequent neoplastic transformation. This argument predicts an increase in the incidence of new clones of transformed cells with increasing intervals between relapse. Cytogenetic analyses have already been reported in CML that suggests that this may be the case (Fig. 1). Additional and more dramatic evidence was recently provided by Fialkow et al.<sup>46</sup> Male sibling bone marrow was transplanted into an untreated female with acute leukemia. Ten weeks later leukemic cells were found in abundance, and the cells were male

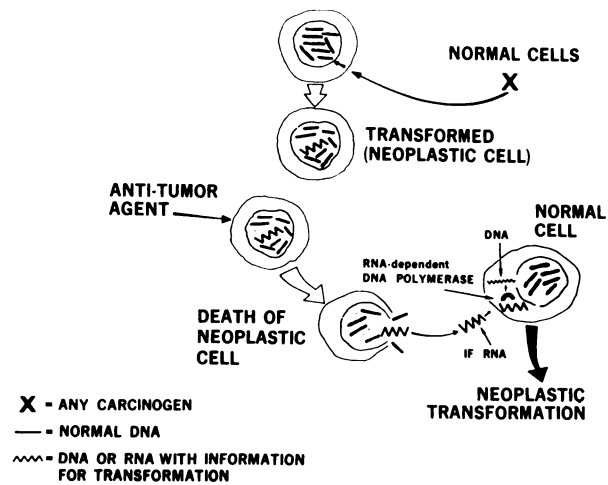
**Fig. 1.**—Incidence of changes in karyotype of leukemic blast cells as function of time between relapses. All patients were originally Philadelphia chromosome positive. Cytogenetics analyses were performed when patients were in an acute blastic crisis. Cytogenetic analyses were performed by Dr. J. Whang-Peng, NCI, NIH.



in karyotype. These findings strongly suggest that relapse might have occurred by genetic retransformation. It is obvious that if this is the case (and, if, as in other mammals, an RNA virus is involved in the etiology of human leukemia), selective polymerase blockade might help prevent these relapses. Perhaps in some instances relapse is even facilitated by chemotherapeutic agents by liberating the agent in question from transformed cells (Fig. 2).

*Rifamycin SV, Rifampicin, and Rifampicin Derivatives:* Since rifampicin was a known potent inhibitor of bacterial DNA-directed RNA synthesis (RNA polymerase) but not of mammalian RNA polymerase and since some bacterial mutants resistant to rifampicin showed changes in RNA polymerase, it was clear that antibiotics in this class could be regarded as potentially specific in their interactions with various polymerases. Therefore, it was of obvious interest to determine if rifampicin or any of its derivatives (Fig. 3) inhibited RDDP activity. Gurgo et al.<sup>43</sup> found that N-demethylrifampicin and a dimethyl benzyl derivative of rifampicin (but not rifampicin itself) inhibited RDDP of three different RNA tumor viruses but did not inhibit *E. coli* or KB cell DNA polymerase at similar concentrations. Similarly, we found that N-demethylrifampicin but not rifampicin<sup>31</sup> inhibited RDDP of human

**Fig. 2.**—Scheme for possible “reinfection” of normal clones by an inciting agent here given as RNA species (or intact RNA tumor virus). If RNA species (rather than intact virus) can transfer information to new cells, it would theoretically require presence of RNA-dependent DNA polymerase in normal cells. In this case RNA would be precursor of oncogenic proto-virus. In some cases this information transfer could be facilitated by antitumor agents.



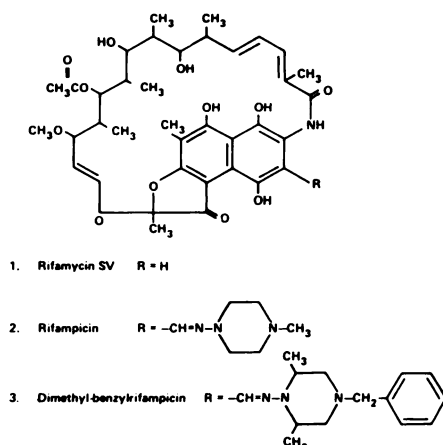


Fig. 3.—Structure of rifamycin SV and derivatives.

leukemic cells but did not inhibit the DNA-directed reaction.<sup>32</sup> However, of rifampicin (2,6-dimethyl-4-N-benzyl-demethylrifampicin) (unpublished results). Furthermore, we are not certain that the selectivity of the N-demethylrifampicin is due to a greater binding to one polymerase versus another. Instead, our findings indicate that when the enzymes are equally purified, the inhibitions are comparable if the same templates are used, i.e., it may selectively inhibit the RDDP because it interferes much greater with binding of RNA to the enzyme than it does with DNA. This appears to be the case with the majority of the 200 rifampicin derivatives we have investigated that showed some selectivity.

Recent results from our laboratory indicate that some rifampicin derivatives and rifamycin SV itself show greater inhibitory effects on the viability of intact leukemic cells compared to normal leukocytes (ALL vs. PHA stimulated normal lymphocytes and CLL vs. nonstimulated normal lymphocytes).<sup>47</sup> Whether these effects are due to a greater effect on leukemic cell polymerase(s) is not known, and it is conceivable that the antileukemic effects have little or nothing to do with inhibition of polymerase activity.

*Streptovaricins:* Brockman et al.<sup>48</sup> recently showed that the chemically parent compound, rifamycin SV, and another group of antibiotics with macrolide "backbone" structures, the streptovaricins, inhibited MSV RDDP. We have found these compounds inhibit RLV polymerase, have only slight effects on human RDDP, but do have "antileukemic" effects *in vitro*.<sup>47</sup> *In vivo* antitumor effects have also now been reported.<sup>49</sup> A word of caution should perhaps be made at this point. Some of these compounds and the rifampicin derivatives are labile, particularly in solution, and different preparations contain varying degrees of impurities. We have found marked variation in results with different lots or when the compound was left in solution more than 1–2 days.

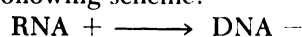
*Miscellaneous:* The effect of some other compounds on RDDP activity have also been studied. For instance, ethidium bromide and, to a lesser degree, daunomycin are inhibitors (Hirschman, S., personal communications). It will be important to determine the mechanism of action and the specificity of in-

hibition in each case. The important inhibitors will likely be those that show *selectivity* and work by binding specifically to the virion RDDP, rather than by interacting with nucleic acid templates.

Another interesting therapeutic approach involves the use of defective viruses. For example, a mutation producing an altered and inactive RDDP may result in a defective RNA tumor virus no longer capable of transforming cells. This appears to be the case, for instance, in the mutant described by Hanafusa.<sup>27</sup> Advantage might be taken of virions with defective polymerases, since they may compete with RDDP from an "active" RNA tumor virus for the RNA of the active virus, in effect acting like a therapeutic chelating agent.

#### RNA-DIRECTED RNA SYNTHESIS: SECOND PATHWAY OF RNA TUMOR VIRUSES?

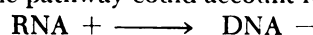
It is possible that integration and replication of the genome of RNA tumor viruses involve more than the pathway of reverse transcription. Green et al.<sup>50</sup> recently reported detecting minus strands of RNA (RNA -) in MSV-transformed cells. The pathway of reverse transcriptase may account for + strands by the following scheme:



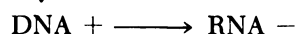
↓



The same pathway could account for RNA - by:



↓



Levinson and Bishop et al.<sup>26</sup> have found that only 5% of the RNA genome is converted to double-stranded DNA. Perhaps not all of the viral genome is integrated into host DNA by the reverse transcriptase pathway. An alternate pathway may exist involving direct conversion of RNA+ → RNA-, i.e., an RNA-directed RNA synthesis. However, an RNA-dependent RNA polymerase has only been found in bacterial RNA viruses and in nontumor animal RNA viruses, but there could be a cellular enzyme, playing a role in transcription of certain species of RNA in normal cells, and RNA tumor viruses could be capable of utilizing the enzyme from cells. It could be that the reverse transcriptase is the only pathway involved in neoplastic transformation, while an RNA → RNA pathway may also be involved in viral replication. If so, then agents that specifically bind to this type of polymerase should be looked for as another class of antiviral compounds.

#### IMPLICATIONS OF RDDP OTHER THAN IN NEOPLASIA

##### *Gene Amplification and Cell Differentiation*

The ability of virion RDDP to catalyze synthesis of DNA complementary to virus 70S RNA immediately raises the question whether other RNAs may serve as templates for this enzyme or other cellular polymerases. As mentioned before, we have shown that some cellular cytoplasmic RNAs may serve as templates for cellular RDDP<sup>32,35</sup> as well as certain phage RNAs (Sarin, P., Smith, G., and Gallo, R., unpublished results). Similar results (with cytoplasmic RNA) were reported almost simultaneously by Cavalieri with *E. coli* DNA

polymerase, and subsequent similar findings showing that cytoplasmic RNAs may function as templates for RDDP in mouse leukemic cells have been reported by Bosmann.<sup>37</sup> It should be noted that although the reports on cellular RDDP have shown activity with cellular RNA templates, in no case as yet has complementarity been demonstrated between the RNA and the product DNA. It is possible that only small portions of the RNA are copied. However, if synthesis of DNA complementary to cellular RNA templates can be achieved, it is clear that theoretically a tool will be at hand for rapid synthesis of specific genes.

By a different approach, Tocchini-Valentini<sup>51</sup> has reported suggestive evidence for cytoplasmic RNAs (rRNA) acting as templates for cellular RDDP. He has shown that the amplification of genes for rRNA, which normally takes place in differentiating oocytes of *Xenopus*, is blocked by an RDDP inhibitor, namely the dimethyl benzyl derivative of rifampicin. From these results it is reasonable to speculate that amplification of rRNA genes develops from a reverse transcriptase mechanism in embryonic cells. However, as the authors noted,<sup>51</sup> this concept is based on the premise that the inhibitor is specific for RDDP, as suggested by the findings of Green et al.<sup>50</sup> However, as noted above, this may not be the case. Therefore, although an extremely important and attractive idea and one presently under test in many laboratories, there is no direct in vivo evidence to support it at present.

The idea that a RDDP is operative in normal cells and plays a key role in cytodifferentiation is an essential component of Temin's provirus hypothesis (see below). This attractive theory is testable in some respects, and it may stimulate as much research activity as any previous proposal in biology in the last decade. As described above, the reports of *cellular* RDDP that utilize *cellular* RNA as templates and the inhibition of gene amplification by the dimethyl benzyl derivative of rifampicin are (at minimum) in keeping with the hypothesis.

#### *Information Transfer*

Another critical component of the provirus hypothesis (in regard to cell differentiation) involves transfer of information from one cell to another, particularly of RNA species or RNA-protein complexes, which will be templates for RDDP of the accepting cell. Although there is no evidence for uptake of mRNA by mammalian cells, there are reports of uptake of crude whole RNA<sup>52,53</sup> and of tRNA.<sup>54</sup> However, as yet no one has reported conclusive evidence that the RNAs taken up actually function in the cell.

#### *Antibody Production*

It is apparent that a reverse transcriptase mechanism might explain some aspects of the secondary immune response. After induction of the transcription of a specific new species of RNA following antigenic stimulation, the appropriate antibody is synthesized. The RNA or a ribonucleoprotein complex may then pass to other cells and insert information (perhaps previously lacking in these cells) or amplify genomes for this species of RNA through an RDDP.



The transcription of these genomes will then occur subsequent to a second antigenic challenge.

#### RECENT THEORIES OF ONCOGENESIS INVOLVING RNA TUMOR VIRUSES

Two interesting theories of oncogenesis with unorthodox roles for tumor viruses have recently been proposed.

##### *Oncogene Theory*

The main tenets of this idea, originally proposed by Huebner and Todaro<sup>55</sup> and since extended by Huebner,<sup>56,57</sup> are that many, if not all, vertebrates contain the genetic information for producing portions or all of C-type RNA viruses. This information ("virogene") is vertically transmitted, i.e., from one generation to the next and from cell to daughter cell. This "virogene" includes a portion responsible for neoplastic transformation (oncogene) and presumably is expressed (at least in part) in undifferentiated proliferative cells (e.g., early embryo) but not normally in adult mature (nonproliferative) cells. Exposure to carcinogens, irradiation, and perhaps other "tumor viruses" (such as DNA tumor viruses or other RNA viruses) and the host genotype itself determine if activation of this genome will occur. Activation will lead to derepression for information normally under repressor control, and depending on many factors whether virus production, neoplasia, or both, develop.

As noted by Temin,<sup>58</sup> the oncogene is an epigenetic theory, fundamentally based on the idea that a regulatory switch mechanism controls a stable alteration in genotype.

Although none of the findings listed below are evidence *for* the oncogene theory as opposed to alternate possibilities, they are *all* in keeping with the theory. (1) Cell culture experiments have shown that under certain conditions normal embryonic cells may spontaneously transform to type-C virus-producing neoplastic cells. (2) Seroepidemiologic studies have shown that antigens thought to be specific for RNA tumor viruses are present in tissues of many vertebrates in early embryogenesis.<sup>57</sup> (3) Cancer of mammals, as it naturally occurs, is associated generally with C-type particles rather than DNA viruses. (4) Many biochemical studies have revealed that proteins and RNA species present in tumor cells (sometimes initially thought to be unique to neoplasia) are usually present in fetal tissues at some stage of development,<sup>30</sup> indicating that in the process of neoplastic transformation genomes repressed in adult tissues but active in fetal life are reactivated. (5) Several studies have shown that C-type RNA virus group-specific antigens or the C particles themselves appear with chemical or radiation-induced neoplasia.<sup>59</sup>

##### *Protovirus Hypothesis*

Temin's imaginative theory proposes, among other things, a mechanism for somatic cell genotypic variation and gene amplification with their inherent implications to cell differentiation and growth, as well as providing a mechanism for secondary antibody response, the origins of RNA leukemia and

sarcoma viruses, and neoplastic transformation. In common with the oncogene theory the origin of the C particles are from cellular genes, portions of which at least include "protovirus." The latter arise as products of a cellular RNA template and RDDP. Altered or abnormally integrated protoviruses lead to oncogenesis (this could occur from changes in RNA, DNA, or the polymerases). How the carcinogen would do this is not answered by the theory, and, of course, neither this nor the oncogene theory accounts for the mechanism of production of the cancer from the altered DNA.

The observations (listed above) in keeping with the oncogene theory are all compatible with the protovirus theory. In contrast to the oncogene theory Temin's proposal (1) combines viral and *somatic mutation* ideas rather than regulatory switch mechanisms; (2) the *central* key to the theory is RDDP and it must be present in at least some normal cells and particularly embryonic cells; (3) requires transfer of information, presumably RNA.

Since the theory is too new for detailed evaluation and it has very recently been described by its originator,<sup>58,60</sup> a vigorous account here is not needed. Suffice it to say that some aspects are already of great importance in neoplasia, and in its total content it may represent one of the unusual moments in biology when a novel approach opens avenues in every sphere of biology.

#### CONCLUSIONS

The presence of an RNA-dependent DNA polymerase or reverse transcriptase has been found in every RNA oncogenic virus investigated, with the exception of some mutant viruses that have *lost* the ability to infect or transform normal cells. It is assumed that the role of the enzyme is to convert viral 70S RNA to a DNA copy, allowing the viral genome to be inserted into host cell DNA.

Nononcogenic viruses either contain no polymerase activity or an RNA-dependent RNA polymerase with the exception of the Foamy viruses which have been shown to contain reverse transcriptase. However, it is thought that this type of virus may eventually be shown to be oncogenic. With care to avoid a number of pitfalls, demonstrating the presence of a true reverse transcriptase in an isolated particle would strongly support the notion that the particle is an RNA virus and potentially oncogenic. Since there is sometimes serious controversy over whether particles isolated from human tissues are viruses, let alone oncogenic, the potential practical usefulness of this molecular biological approach is obvious, and it has already been adapted to this situation.

The presence of a true reverse transcriptase (catalyzing synthesis of DNA from *pure single stranded natural RNAs*) in cells is more difficult to establish. The use of some synthetic templates such as the RNA-DNA hybrids poly rA.dT to detect the presence of reverse transcriptase has led to some confusion. Although reverse transcriptase has great affinity for these templates *they lack specificity*, i.e., virtually any DNA polymerase will use them. Thus, activity with these templates does not in itself indicate that reverse transcriptase is present. At this time there is no published definitive information demonstrating the presence of this enzyme in any normal cell. An unequivocal demonstration of its presence in a human tumor tissue would, therefore, appear to indicate that information from an RNA oncogenic virus may be present.

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