RNA Tumor Viruses and Leukemia: Evaluation of Present Results Supporting their Presence in Human Leukemias

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I. Introduction

Type-C RNA viruses have been isolated from many species. In several, they have been often associated with leukemia and shown to reproduce the disease on inoculation into recipient animals. In a few species the data appear now to be conclusive that they are the major cause of the natural disease. Two major difficulties in verifying results that the virus causes the disease in some animal systems have been: (1) the long latent period for evident disease, and (2) the fact that many type-C viruses are apparently not oncogenic. Regarding the latter, we have argued for a major subdivision of these viruses based on a molecular hybridization assay (see below).

II. Class 1 and Class 2 Viruses

We isolate RNA from the virus and make it radiolabeled and then hybridize it to excess DNA purified from uninfected normal tissues of the animal believed to be the natural host (1, 2). By this assay, the RNA of some viruses hybridizes virtually completely to the cell DNA and the quality of the hybrids are good (judged by measuring the temperature required to dissociate the RNA-DNA complex). We call these viruses class 1 (1). There is usually data (besides the hybridization results) which indicates that these viruses are really normal cell gene products. We believe that they carry out some normal function, probably during development (3–6). In addition, it is possible that they play an important evolutionary role in transmitting information horizontally between different species, information which may subsequently be of advantage to the recipient species and become transmitted vertically (parent to progeny) in the germ line of that species. In this case, it becomes part of the normal genetic elements of the recipient. In this respect, Todaro and his colleagues have provided evidence for interspecies transfer of virus from a

primate to certain cats (7; and see Todaro elsewhere in this workshop). This is now the feline endogenous virus, RD114.

Examples of class 1 viruses include: RAV_0 of chickens; the guinea pig endogenous virus; the xenotropic endogenous viruses of mice; RD114, the endogenous virus of cats; and the only isolate to date of an endogenous virus from primates, the baboon endogenous virus (BaEV) (see next section).

The RNA of other type-C viruses show only limited hybridization to the DNA of uninfected normal cells of the persumed host. These we call class 2 (1). As a rule they are oncogenic. Examples of these are: avian myeloblastosis (AMV) and sarcoma viruses of chickens (AvSV); the leukemia and sarcoma viruses of mice (MuLV, MSV); the leukemia and sarcoma viruses of cats (FeLV, FSV); and the leukemia-sarcoma viruses isolated from primates, the gibbon ape leukemia virus (GalV) and the woolly monkey (simian) sarcoma virus (SSV). I think it is because of the genetic differences of these viruses compared to their host combined with their ability to add this *new* information to the DNA of the host, that gives them their oncogenic capacity. Although it is not yet known, I suspect the bovine leukemia virus (See A. Burny elsewhere in this workshop) will also be a class 2 virus.

Most of the class 2 viruses are infectious for the species they produce neoplasias under natural conditions. The exact route of infection is not clearly known, but in the case of AMV the leukemia is thought to occur most commonly from congenital infection (8), while in cats it appears to by contact between animals and possibly by congenital infection (9). It is important to note that many *normal* animals may contain class 2 viruses. This has given rise to mouch confusion. It does not mean the virus is endogenous in the genetic or molecular sence. For instance, many normal cats get infected and do not get disease. It must depend on other factors, e. g., genetic resistance or susceptibility to activation, immune response, or possibly fine variation in the genetic composition of the virus.

If class 1 viruses are gene products, what is the origin of class 2 viruses? We believe class 2 viruses are derived from class 1 viruses by genetic change in the class 1 viruses when the latter escape host control. This can occur when it becomes infectious for its own host and by processes not understood undergoes genetic change. The "new" virus is different from the original and on its way to becoming class 2 (see Fig. 1). In this case, a class 1 and class 2 virus will be related (by antigenic tests of their proteins and by nucleic acid hybridization). Thus, AMV is related to RAV_0 and MuLV and MSV are related to the xenotropic murine endogenous viruses. In these cases then we think the class 2 viruses evolved from the class 1 viruses. In contrast, in cats FeLV and FSV are not related to RD114, yet there is considerable (20–40 %) of the information in FeLV which is related to normal cat DNA (10). In this case, I suspect that FeLV evolved from *another* class 1 virus of cats, different from RD114. In the case of primates, like in cats, the class 2 viruses (GaLV and SSV) are not related to the class 1 virus, the endogenous virus of baboons (BaEV). However, unlike the situation with FeLV, FSV, there is almost no hybridization of the RNA of GaLV and SSV to DNA from normal primates (11). We suspect that GaLV and SSV were derived from another species, and Todaro and his colleagues (12) and our laboratory (11) have provided evidence that these viruses originated from a mouse (see dashed line pathway, Fig. 2). This is analogous to the above mentioned acquisition of RD114 by certain cats from a primate

(see solid line pathway in Fig. 2). However, apparently in the case of SSV and GaLV there has not been integration into the germ line of primates. Instead, the virus has become a horizontally moving infectious agent of primates (see next section). In contrast, in the case of RD114 after acquisition from a primate estimated to have occurred in the distant past (7), the virus apparently integrated into the germ line and became part of the normal genome of certain cats (class 1). Thus, it appears that if a class 1 virus escapes host control and enters a new species, it can become either an infections agent for the recipient species (a class 2 virus) or it can become endogenous to the recipient species (class 1) (see Figs. 1 and 2).

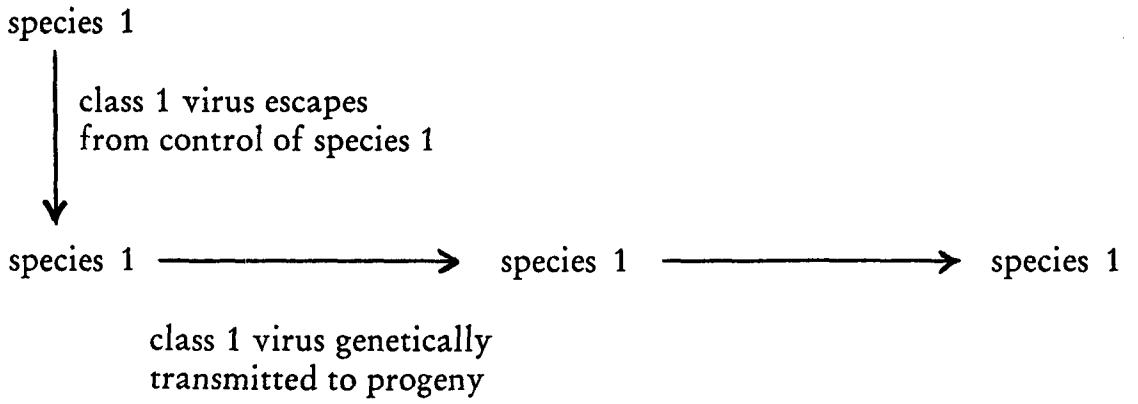

Fig. 1: Schematic illustration of the transmission of a truely endogenous type-C virus (Class 1 virus) from one animal to another animal of the same species. Normally, the class 1 virus is under host control and is vertically transmitted in the germ line. Somehow the virus has escaped from this control, and it is transmitted to other animals. How it escaped control is not known. Perhaps this involves minor genetic change in host in virus, or in both. Once it becomes transmitted horizontally it undergoes some slight genetic change.

Infects only same species, but by mechanisms not understood some genetic change in viral genes occur, and virus becomes a class 2 virus for species 1, although still highly related to its class 1 precursor virus, differences exist. Example: AMV from RAV_0; MuLV from murine xenotropic endogenous virus.

A second mechanism for formation of a class 2 virus might be by direct genetic alteration of the endogenous class 1 virogene followed by expression and formation of virus with new genetic components. For instance one can envision the class 1 virogene of the normal animal as a "hot spot" for mutation by chemicals or radiation or a "hot spot" for receiving new information as when a DNA virus infects a cell. This is the so called "Virus Hot-Spot" proposal outlined in more detail at this workshop in 1973 (4). In this case, the virus did not cause the disease, but it was a pruduct of the disease. However, once formed, it could transmit the oncogenic information to other cells in the same animal. If it escapes host control it might be transmitted to other animals of the same species or to another species, and in these instances it can become a primary cause of the natural disease. Some of these concepts are summarized in Fig. 3.

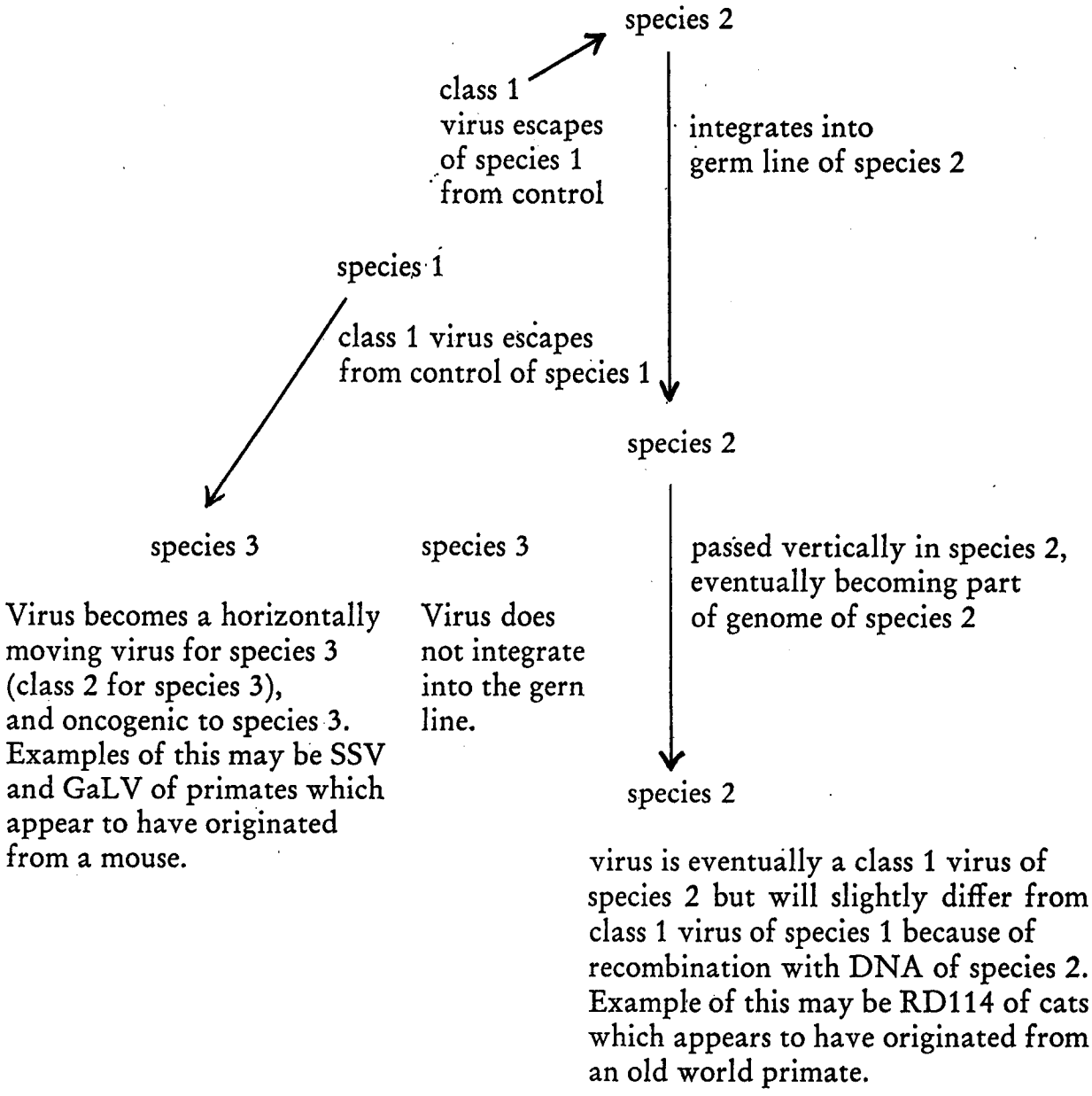

Fig. 2: Interspecies transmission of type-C virus. There is now evidence that type-C viruses can be transmitted from one species to another. As illustrated, in some instances a class 1 virus of one species (here called species 1) escapes from host control, enters a new species and becomes integrated into the germ line of the new species (here called species 2). Presumably, it offers a selective advantage to species 2 because it is maintained and eventually becomes part of the normal cellular genome of species 2. In this example, the virus was initially class 1 for species 1 and class 2 for species 2, but it eventually becomes a class 1 virus of species 2. Todaro and colleagues have provided the first example of this in providing data for transmission of a primate type-C class 1 virus to cats. This virus apparently became the endogenous cat virus known as RD114. Alternatively, the class 1 virus of species 1 may not integrate into the germ line and not be maintained in the new species (here called species 3) but instead persists as a class 2 infectious virus horizontally transmitted from species 1 to species 3 and between members of species 3. This may be the origin of the primate type-C infectious class 2 viruses, SSV and GaLV which appear to be capable of moving horizontally among some primates and which may have originated from a rodent.

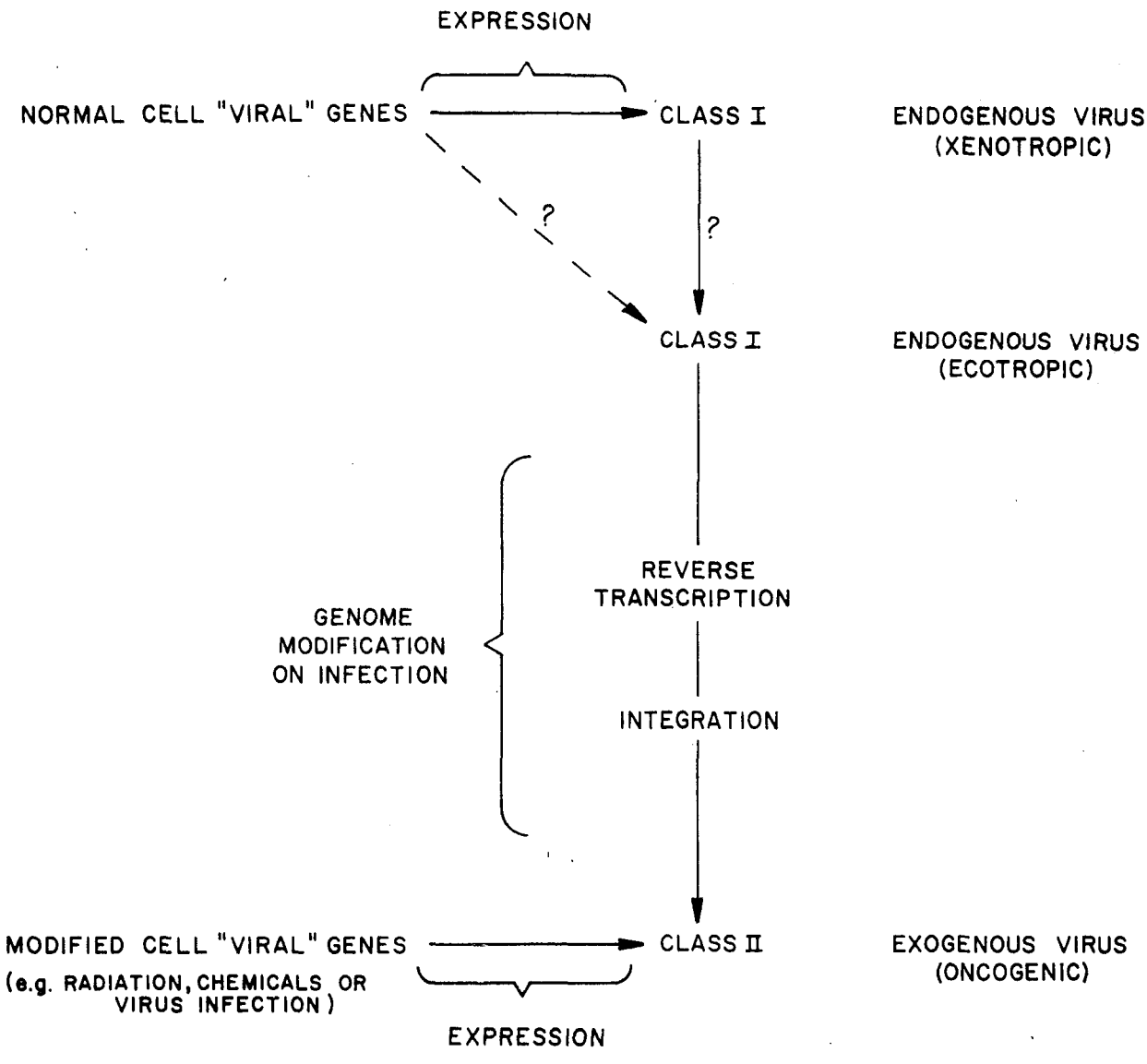

Fig. 3: Hypothetical scheme for origin of a class 2 virus. The model like other theories proposes that type-C viruses originate from normal cell genes. Complete expression leads to formation of the class 1 endogenous virus which is not able to infect most or all cells of the host it originates from (xenotropic virus). Other endogenous viruses are ecotropic, i.e., they can infect cells of their host. I believe these viruses evolved from the xenotropic class 1 viruses by minor genetic change. Once they become infectious more substantial genetic change may occur leading to the formation of a class 2 virus which is often oncogenic. An alternative route for formation of a class 2 virus is by direct mutation (e.g. by chemicals or radiation) of a "hot spot" within the class 1 viral genes of the normal cells. If this occurs and it is followed at some later stage by complete expression, the consequence should be creation of a class 2 virus, i.e., a virus with at least some genetic difference from the normal host. This hypothesis "virus-hot spot" theory has been discussed in more detail previously (4).

III. The Primate Type-C RNA Tumor Viruses

As discussed above, type-C viruses have been isolated in recent years for the first time in primates. The first was isolated (13) from a woolly monkey, a household pet of a woman living in California. The animal developed a spontaneous fibrosarcoma of the neck which yielded the virus. To this date there is only *one* isolate of the "woolly monkey" virus which has become known as the simian sarcoma virus (SSV). Almost simultaneously viruses were isolated from gibbon apes with various hematopoietic neoplasms, especially myelogenous and lymphoid leukemias (14). Of great interest, these isolates first came from a colony of 195 gibbons, 103 of whom were *injected with blood from humans with malaria.* The control group of 92 animals were not inoculated (15). *All 10 neoplasms developed in the inoculated animals.* Virus was isolated only from the animals with neoplasms. Recently, 3 isolates of the gibbon ape leukemia virus (GaLV) were obtained from brain extracts from "normal gibbons", but 2 of the 3 came from animals injected with extracts of brains from humans with Kuru (16), and the 3rd was a cage-mate of the other two. There have been a few isolates from gibbons not injected with human material, but *all* had close contact with man. It was surprising that despite the fact that SSV came from a new world monkey and the gibbon isolates from an old world ape, the various GaLV isolated and SSV ere extremely closely related.

When injected into some recipient primates SSV has produced sarcomas and malignant brain tumors (13, 17). Injection of GaLV into young gibbons has produced in some instances myelogenous leukemia (18). More importantly, apparently GaLV can spread horizontally among gibbon cage-mates and produce leukemia in animals that do not develop a sufficient immune response (18).

I believe that the above studies are of unusual importance: *first* because they are the first isolates of type-C viruses from any primate; *second,* because the viruses are proven oncogenic; *third,* because they provide the closest model of animal neplasias for man we have; and *fourth,* because I strongly suspect that these repeated relationships to man are not mere coincidences. Although the origin of these viruses may have been from mice (12, 11), it is clear that now they have become infectious agents among primates. For the reasons descibed above, combined with results I will describe below, I believe that these viruses form a family of closely related class 2 type-C viruses infectious for primates, including man, and capable of inducing neoplasias among them.

IV. Assays for Type-C Virus Markers in Human Leukemia and Evaluation of Results

Although the earliest evidence for type-C virus in human leukemia came from pioneering electron microscopic studies, particularly by Dmochowski and his associates (e. g., see references 19 and 20 for reviews by Dmochowski, it is clear that if viruses are involved in the leukemias of man, fully formed, released, and infectious virus must be a very rare event. Virus if present, must be at a very low level, or defective (i. e. unable to replicate and therefore not generally detectable by microscopy or bioassays) or be suppressed (i. e., full provirus is present in at least some cells but is not fully expressed). For these reasons we turned to molecular

biological and immunological approaches to determine if markers of type-C virus could be detected in human leukemias. There are 5 viral related molecules or structures which have been described in some human leukemic cells: (1) reverse transcirptase; (2) viral related nucleic acids; (3) p30 protein; (4) membrane protein related to gp 70; (5) cytoplasmic particles with similarities to intracytoplasmic virus.

Reverse transcriptase (RT). Evidence for a DNA polymerase in some human leukemic cells with properties like the viral enzyme began in 1970 with a report by Gallo *et al.* (21) describing this activity in 3 patients with acute leukemia. Later this enzyme was partially purified, shown to be biochemically distinct from the major 3 DNA polymerases of uninfected cells (22–26), and localized to a cytoplasmic particulate fraction of the cell (22–26). Also during this period, the properties of viral RT became known in detail, and it was possible to set up certain criteria for calling samples positive for RT. Table 1 lists those criteria used in our

Table I: Criteria for Reverse Transcriptase in Human Leukemic Cells

1. found in cytoplasmic particulate fraction.
2. carries out endogenous RNase sensitive, actinomycin D resistant DNA synthesis.
3. in some cases it can be shown that the DNA is covalently attached to an RNA primer and hydrogen bonded to an RNA template.
4. after partial purification (and if free from significant contamination with cellular DNA polymerases) RT will show strong preference for poly A·oligo dT compared to poly dA·oligo dT as template-primer.
5. utilizes poly C·oligo dG as template-primer efficiently and this is a relatively specific template-primer for RT.
6. the molecular weight is about 70,000.
7. immunologically closely related to RT from wooly monkey (simian) sarcoma virus and gibbon ape leukemia virus.

laboratory. The detailed assays will not be described here since they have been included in recent reviews from our laboratory (26). Detection and characterization of this polymerase in extracellular viruses is simple, but it is very difficult to detect small amounts of RT in cells. This is due to the presence of proteases, nucleases, and most importantly, interference and confusion in the assay by the presence of the much greater amounts of the cellular DNA polymerases, DNA polymerases α, β, γ and the mitochondrial DNA polymerase (27). In my judgement, there is no really simple assay for RT in cells which gives confidence that one is actually measuring RT. The most sensitive and simplest assay I know of is the "simultaneous detection" assay for RT and high molecular weight RNA developed by Spiegelman and his group (see ref. 28 and Spiegelman in this volume for details of this assay). In this approach after isolation of the cytoplasmic particulate fraction from the cell, DNA is synthesized from nucleic acids native to the same fraction by simply incubating this fraction (containing nucleic acids and polymerase) in the presence of the substrate nucleotides (dATP, TTP, dCTP, dGTP), one or more of which are radiolabeled, in the presence of appropriate buffers. The reaction is shown to be at least partially sensitive to RNase and resistant to actinomycin D. Product anal-

ysis is then performed, and the newly synthesized DNA is shown to be joined to a large RNA molecule. These results, taken together, suggest that the template is a high molecular weight RNA (hence viral-like) and the reaction RNA directed (hence like reverse transcriptase). Using this assay, the results indicate that most and perhaps all leukemie cells contain this complex. This has been confirmed by our laboratory (29) and by Mak *et al.* (30). An additional factor supporting the conclusion that this assay detects reverse transcriptase and viral-like RNA is the failute by Spiegelman's group to detect positives in normal human tissues (28, 31). The strengths of this approach are its sensitivity, simplicity and capacity for application of relatively small quantities of tissue. Its weakness is the possibility of giving false positive results.

Greater assurance that the enzyme is, in fact, RT comes only from purification and biochemical characterization of the polymerase (22, 25, 32), followed by immunological (32, 24, 25, 33) comparisons to known RT from animal RNA tumor viruses. This is the approach used in our laboratory, and by this approach our positive samples are limited to about 20 % of patients with leukemia. Moreover, our samples with patients in remission have uniformly been negative. In the case of adult AML and to date *only* in this patient population, when RT was found and purified, in every case it was antigenically closely related (possibly identical) to RT from SSV. We now have 8 cases of AML where this has been demonstrated (32, 24, 25, 33), but even in adult AML we have found this in a fraction of the patients studied.

The advantage of this approach, of course, is that when a positive is scored, one has assurance it scores for viral reverse transcriptase. The disadvantages are its complexity, time, requirement for larger amounth of tissue, and the greater likelihood of false negatives. We think to detect and verify RT in leukemic cells that it is essential to utilize the cytoplasmic particulate function. Essentially, this is a microsomal-membrane fraction but it includes some mitochondria. In our experience, it is almost essential to use fresh cells. For details the reader is referred to four recent papers from our laboratory (25, 24, 32, 33) and recent method reviews (34). *Viral Related Nucleic Acids.* Three characteristics have been used for determining a nucleic acid found in leukemic cells is viral related: (1) size; (2) in the case of some RNA molecules, their association with (RT; (3) nucleotide sequence composition. The latter has been estimated by the technique of molecular hybridization. We have already touched on the first two. Viral-like size is high molecular weight, i. e., about 35S or a 70S complex. This was first demonstrated by Spiegelman's group in leukemic cells by the indirect simultaneous detection assay described above (31) and confirmed by us in a few patients (29, 35). Subsequently, Mak *et al.*, provided evidence for 70S RNA by pulsing cells with ^{3}H-uridine and isolating cytoplasmic particles released into the media in short term culture containing labeled 70S RNA (30).

Association of the RNA with RT is shown by assaying the endogenous RT reaction of the cytoplasmic particulate fraction and determining that the DNA synthesized is associated with a large RNA molecule (31, 29, 30, 35).

The last characteristic, nucleotide sequence composition, is perhaps the most important. It is designed to determine if a given nucleic acid in the cell contains sequences related to a known type-C RNA tumor virus. The approaches that have

been used to obtain this information are listed in Table 2. The advantage and disadvantage of each will be discussed separately, and the results summarized.

1) *Viral cDNA to Cell RNA.* In this approach viral cDNA labeled probes are prepared by carrying out endogenous DNA synthesis with disrupted virus. The virus may be any test animal RNA tumor virus. The cDNA represents a transcript of the viral genomic RNA. The cDNA is radiolabeled because one or more of the deoxyribonucleoside triphosphate substrates are radiolabeled. The cDNA is then hybridized to RNA purified from the leukemic cell. Various hybridization assays may be employed, e. g., analysis of hybrids by cesium sulfate centrifugation or by S1 nuclease digestion (a nuclease specific for single stranded nucleic acids, leaving undigested and hence TCA precipitable the hybrid molecules). Details of these procedures have been summarized in a recent review (36). The advantages of this technique are its simplicity, speed, and capacity to generate high specific activity probes. The disadvantages are that the cDNA made are small (4S or smaller), and although it is possible to make cDNA which represents the entire viral RNA genome, it is generally a very unbalanced copy. In other words much of the cDNA is only from a small fraction of the viral RNA. Therefore, it is a limited probe. Another disadvantage is that it only looks at the cell RNA. A negative result does not mean that the viral information is not present; it only suggests that it is not detectably expressed. It may ba present in the cell DNA but remain unexpressed.

Hehlman *et al.* used this approach to detect sequences in most or all human leukemic cells related to RLV (37, 28). They found none related to AML or MMTV. Thus, there was some specifity. They did not find these in the normal cells they examined. However, the amount of hybridization was low. Questions arise concerning the interpretation of these results. Are they really viral or simply sequences in cells related to viral sequences? Therefore, the specifity pattern (i. e., to which virus) becomes very important. The negative data with AMV are really not so helpful since on evolutionary basis we expect more sequences in human cells related to mouse than to birds. Regarding, the negatives in normals, again as mentioned above, this approach does not mean they are not there. It means only they were not expressed at a level detectable by the assay. In this regard, using the same approach, we have found RLV related sequences in normal PHA stimulated human lymphocytes (38). A. Tavitian elsewhere in this workshop has used this approach with MuSV and SSV (39). He finds hybridization with the cDNA from SSV and with cDNA from the murine virus to leukemic cell RNA (39).

In summary, unless a major degree of hybridization is obtained with a given viral cDNA probe and cell RNA and good quality hybrids are obtained, results of this kind can not prove the presence of added viral information. They do show that viral related sequences are present; they are at least consistent with the viewpoint that virus is present; and as the simplest and earliest approach used, they provided impetus for further experiments.

2) *Viral cDNA to Cell DNA.* The probe (labeled viral cDNA) is the same as that described above. However, instead of cell RNA, cell DNA is used. This, of course, examines not only what is expressed, but unexpressed genetic information. To my knowledge, there are no published reports using this approach with human leukemic cells. Recently G. Aulakh in our laboratory did apply this with cDNA from RLV, and he has detected DNA sequences *distally* related to RLV 70S RNA

in human leukemic cells (40). However, neither these nor any published results mean that leukemic cells are infected by RLV. They indicate only that leukemic cells contain some nucleic acids with some nucleotide sequences of RLV.

3) *Viral 70S RNA to Cell DNA.* With this method the entire 70S RNA genome of the virus is radiolabeled. The RNA can be radiolabeled by pulsing virus infected and growing cells with ^{3}H-uridine and isolating the labeled 70S RNA from the released virions. This technique and the subsequent hybridization of the 70S RNA to excess cell DNA has been most extensively employed by M. Baluda and his colleagues with AMV and avian leukemia. The advantages of the technique stem mainly from the use of the whole genome which theoretically, of course, is a much more complete probe than the cDNA methods. The disadvantages are that some of the RNA degrades, and it is difficult to prepare the RNA with sufficiently high specific activity as is often needed. The latter difficulty has been circumvented by another labeling technique. In collaboration with W. Prensky at Sloan Kettering, D. Gillespie and C. Saxinger in our group have isolated unlabeled 70S RNA from virus and labeled it *in vitro* with I^{125}. This results in a probe of higher specific activity. We have used this 70S RNA from different viruses to hybridize to human leukemic cell DNA (24). Using this method, we initially did not find extensive hybridization of the RNA of any virus to leukemic cell DNA (24)* (see note added in proof).

4) *cDNA From Human Leukemic Cell Cytoplasmic "Particles" to Viral 70S RNA.* This approach merits special discussion, *first,* because it has been employed in only a few laboratories; *second,* because as the newest method it is not generally understood; and *third,* because unexpectedly, it has given the most interesting positive results, albeit not always easy to interpret. While examining leukemic cells for reverse transcriptase we discovered that the enzyme was located in a cytoplasmic particulate fraction (22–25, 32, 33, 35). Before the enzyme is purified, it is associated with RNA in the "particle" (35). This particulate fraction can be purified and the "particles" treated as if they were virions in the sense that an endogenous DNA synthesizing system can be detected and utilized. Surprisingly these particulate fractions frequently exhibit biophysical characteristics of mature type-C virions (24). This includes apparent morphological integrity in repeated banding in sucrose gradients, size, and density (24, 35). We employed the "particle" isolation approach to obtain reverse transcriptase while Baxt *et al.* first used them to prepare labeled DNA probes (cDNA complementary to RNA in the "particles"). These cDNA probes were tested to see if they contained nucleotide sequences homologous to RNA of some animal tumor viruses. Again, positive results were obtained with RLV but not with AMV or MMTV, Baxt, *et al.* then used this cDNA to provide evidence that leukemic cells contain nucleotide sequences not present in normal leukocytes (41). This was done by hybridizing this cDNA to excess normal cell DNA and finding that a very small portion did not hybridize to leukemic cell DNA. Baxt has subsequently claimed that the extra sequences are related to the RNA of RLV (42). These observations have major implications, but they have not yet been confirmed. It should be emphasized again that these results do not mean that human leukemia is due to infection by RLV. They do argue that extra information is present, most likely the result of addition by a virus which appears to have at least some sequences in common with RLV. We also used this cDNA synthesized from the endogenous reaction of the cytoplasmic

"particles" from fresh human leukemic blood cells (35, 43). Our approach was to hybridize this DNA to RNA from many RNA tumor viruses to determine if there was an "affinity pattern", i. e., did the cDNA probe hybridize to RNA of various viruses in some particular manner? Would it be qualitatively like any of the known viruses? We found: (1) that in the case of myelogenous leukemias the cDNA hybridized significantly more to the RNA of SSV than to any other virus, and (2) the hybridization to RNA from other viruses followed a pattern like SSV, i. e., cDNA from SSV hybridized to RNA from the other viruses with the same relative pattern as the cDNA from the leukemic cells (i. e. to SSV>MuSV >FSV>MuLV>RD114 >FeLV >AMV) (35, 43). These results were confirmed by Mak *et al.* (30).

5) *cDNA from Cytoplasmic Particles of Human Leukemic Cells to DNA of Virus Infected Cells.* This approach uses the labeled cDNA described above (#4) to hybridize to the DNA provirus from tissue culture cells infected with different viruses. We have recently initiated this type of study, and our preliminary results with one patient indicate positive hybridization to cells infected with SSV and more so to cells infected with the baboon endogenous virus.

P30 Protein. This viral internal structural protein is assayed for in cells by several approaches. One method, the complement fixation assay, is thought by some to be the more definitive (R. Gilden, personal communication), but it is not as sensitive as some others. A radioimmune assay (see Todaro elsewhere in this workshop) has been recently widely employed. One approach is to find in cells a protein of approximately 30,000 molecular weight (p30) which will compete in the assay of a known viral p30 and labeled antibody to the test viral p30. It is imperative with this method to show specifity since proteases of molecular weights of approximately 30,000 could give false positive reactions by hydrolyzing the labeled antibody. This appears to have been the case with some suggestions of detection of the p30 of RLV in human leukemia.

Strand and August (43) and Sherr and Todaro (44) have reported detection of a p30 protein related and possibly identical to the p30 protein of the baboon endogenous type-C virus in a variety of human tissues. Since there is now evidence for endogenous (class 1) viral genes in many vertebrate species and since the baboon virus is the only endogenous virus isolated so far from primates, these results were taken to indicate detection of partial expression of a human endogenous type-C virus, i. e., that the p30 protein was a gene product of a putative human class 1 virogene (44, 45). An alternate interpretation in my mind is that they may have found the p30 of the baboon endogenous virus itself, as a consequence of infection of man by this baboon virus (see later section on isolates of primate type-C viruses from human tissues and also note added in proof).

In addition to these results Sherr and Todaro (46) have reported detection of a p30 protein in fresh blood leukocytes of 5 patients with acute leukemia related or identical to the p30 of SSV. Very careful controls were carried out to show specifity. Strand and August have reported similar results but believe this protein to be present more widely in the human population (44). To date Sherr and Todaro have not reported detection of the p30 related or identical to SSV in normal human tissues, but as indicated above Strand and August believe they can detect it in some (44). If they are correct, it suggests rather wide-spread infection of the human population with a virus related or identical to SSV.

Cell Surface Proteins Related to Viral Envelope Proteins. A few groups are examining leukemic cell membrane proteins to determine if any related or identical to envelope proteins (particularly the glycoprotein 70) of specific type-C viruses. Two general approaches are used. (1) Using human sera to determine if there are antibodies directed specifically against a membrane protein of human leukemic cells, and then determining if the antibody also reacts with viral envelope proteins. (2) Purifying viral envelop proteins and determining if antibodies raised against these proteins react specifically with a human leukemic cell membrane protein. Some approaches like these have been used by Metzgar and Bolognesi. They have preliminary data which indicate detection of such proteins on leukemic cells related in some cases to the friend leukemia virus and in others to the primate class 2 type-C viruses (the GaLV-SSV viruses) (47).

Specificity of Viral Markers Found in Fresh Leukemic Blood Cells to SSV and to Baboon Virus. It is clear from the studies described in the preceding sections that many laboratories now with many techniques have found evidence for viral markers (proteins and nucleic acids) in leukemic cells. The problem now is one of interpretation. Do these findings represent detection of normal cell gene products which are leated to viral gene products but have nothing to do with virus? If they represent virus are they only products of an endogenous (class 1 virus) present in all cells (normal or leukemic) in all members of the species and not involved in the disease? What is the meaning of finding markers related to RLV in some laboratories while others find markers related to other viruses? My interpretation of these data is as follows. Type-C viruses of most mammalian species are at least somewhat related by some tests, e. g., it would not be surprising if the genome of RLV contained some nucleotide sequences related or even identical to some in the primate viruses. This is, in fact, known to be the case. No one has published finding markers related to AMV. AMV is not related to the mammalian type-C viruses. If the data are taken together, they strongly imply that it is markers primarily related to the primate type-C viruses which are being found in man, both the SSV-GaLV group and the baboon virus. If SSV-GaLV markers also are detected in some normal tissues of some people, the data are no less significant, if indeed, the findings really represent markers of these viruses rather than related proteins and nucleic acids which are really not viral. As noted earlier the SSV-GaLV group are not endogenous to man in the genetic sense. If they really are in some normal tissues, it must mean that like EBV the virus is widespread in man. This could mean that the situation is somewhat analogous to feline leukemia where the virus, FeLV, infects many cats but produces leukemia in only a fraction (9).

Induction of Exponential Growth of Blood Leukocytes from Patients with Myelogenous Leukemia and Isolation of Type-C Viruses.

Studies with many animal cells indicate that DNA synthesis and cell replication are important for production of type-C virus. Other studies, e. g., with Friend leukemia, indicate that differentiation may also be helpful. In this respect, human AML blood cells are characteristically retarded both in their replication and in their ability to differentiate (4, 5). Hence, when we recently identified a source of conditioned medium (CM) which was produced by a fibroblast strain of a cultured whole human embryo which stimulated prolonged exponential growth of myelogenous leukemia cells in suspension culture (48, 49), we were hopeful that this would

result in the production of detectable *complete* type-C virus by the cultured AML cells. The factor(s) in the CM is heat stable (56 °C for 1 hour) and differs from typical colony stimulating activity (CSA) by many criteria. These include: a) induction of *prolonged* growth in suspension culture; b) growth is exponential; c) it has no effect in the soft agar system, i. e., it does not promote colony formation; d) it is specific for myelogenous leukemic cells having no effect on normal bone marrow cells, CLL cells or ALL cells (49, 50).

We initially treated cells (blood or bone marrow) from 16 patients with myelogenous leukemia. These included 2 remission patients with no detectable (by morphology) leukemic cells. All responded to the CM factor (50). In those with a marker chromosome the marker persisted in culture. These results provide derect evidence for the following conclusions: (1) intrinsic differences exist between normal and leukemic cells in recognition and/or response to some growth promoting factors; (2) similar differences exist between myelogenous and lymphoid cells; (3) myelogenous leukemia cells can be grown *in vitro*; (4) some remission myelogenous cells must still be different from normal myelogenous cells; (5) some myelogenous leukemic cells can be induced to differentiate confirming previous suggestions for this by several groups (e. g., see ref. 57 for review by Sachs).

Cells from several patients (5 of 16) expressed readily detectable viral markers (proteins and/or leukemic cells) specifically related to SSV (50), and cells from one patient (patient A. S. or HL-23) released classical budding type-C virus (48, 49, 51). This virus was transmitted to several secondary cell lines for larger production (51). It is infectious and oncogenic for at least some primates (52). Most importantly, the fresh blood (uncultured) cells of this patient contained RT related to SSV (33), and we and our colleagues have been able to reisolate this virus from the same patient. The original isolate came from passage 10 of her myelogenous leukemic cells obtained from her (pre-treatment) first hospitalization in October, 1973 (see Table 3). Subsequently, virus was isolated in our laboratory and also by G. Todaro from earlier passages of these cells which were kept frozen and later put into culture. These were from passages 5 and 7. We also went back to this original blood sample kept in liquid nitrogen and again isolated the virus (49, 51). In addition, we subcultured the first blood sample at *passage* 1 and isolated the virus again from the subculture (49).

Fourteen months later (December, 1974) when she was in partial remission we received a bone marrow specimen. After only a few passages in culture virus was again identified (by electron microscopy and by reverse transcriptase) (49) and subsequently again isolated by N. Teich and R. Weiss by co-cultivation with a normal human embryonic fibroblast line (51). Finally, a blood sample obtained in December, 1973 (2 months after the first blood sample (now also appears to be releasing virus (unpublished results of P. Markham and F. Ruscetti). See Table 2 for a summary of the isolations.

Type-C viruses related or identical to SSV have now also been isolated twice from a child with lymphosarcoma leukemia by Nooter *et al.* (ref. 53 and see elsewhere in this workshop), by Gabelman *et al.* from a patient with CLL and lung cancer (54), and from a few different human embryos by Panem *et al.* (55).

It should be noted that now there are several reports of isolates of SSV (or an SSV related virus) from man and only one from a woolly monkey (see Table 4).

Table II: Molecular Hybridization Approaches Used to Find Viral-Related Nucleotide Sequences in Nucleic Acids from Human Leukemic Cells*

Labeled Probe	Unlabeled Test Nucleic Acid	Reference for example	Comments
(1) Viral cDNA	cell RNA	28	viral cDNA is from the endogenous RT reaction of virions
(2) Viral cDNA	cell DNA	40	viral cDNA is from the endogenous RT reaction of virions
(3) Viral 70S RNA	cell DNA	24	viral 70S RNA is prepared by pulse labeling virus infected cells with ^{3}H-uridine and then isolating labeled viral RNA or by labeling purified 70S RNA *in vitro* with I^{125}.
(4) cDNA from cytoplasmic particles from leukemic cells	viral RNA	31, 35, 43	This cDNA is a product of the endogenous RT reaction of human leukemic cells.
(5) cDNA from cytoplasmic particles from leukemic cells	DNA of infected cells	42, 38	This DNA of infected cells includes the DNA provirus

* For more details see text.

Some of our isolates in addition to the SSV component also contain a virus related or identical to the baboon endogenous virus. Thus, the virus isolate appears to be a mixture of both types of primate type-C viruses. We have recently learned that the virus isolated from human embryos by Panem *et al.* (56) in addition to having a component related or identical to SSV also has one related or identical to the baboon virus (S. Panem and W. Kirsten, unpublished results).

Although molecular hybridization experiments fail to detect *complete provirus* related to SSV or to our isolates in the fresh cells of this or other patients with AML (23, 37), we have been able to find in her fresh uncultured blood cells the following viral related markers: (1) 35S and 70S RNA (38); (2) reverse transcriptase related to reverse transcriptase of SSV and hence to RT from the viruses we isolated (33, 48); (3) cytoplasmic viral-like particles containing 70S, 35S RNA and the RT (38); (4) cDNA probe synthesized from the endogenous RT reaction of the cytoplasmic particles hybridized to the RNA of SSV, to the RNA of the baboon endogenous type-C virus, to DNA from baboon virus infected human cells but significantly less to DNA from normal human cells (38).

Reasons for Confidence that Virus came from Patient HL-23 not as a Contaminant

Table III: Cell specimens from HL23 and Virus isolates

Cell specimen	Date received	Nature of specimen	Extracellular virus detected by[a] RT	EM	Comments
HL23–1[b]	10/16/73	peripheral blood	pass 5	pass 10	virus = HL23V-1 transmitted to secondary cells
HL23–2	10/16/73	peripheral blood[b]	pass 3	pass 3	same original blood sample as specimen 1
HL23–3	10/02/74	peripheral blood	NT	NT	cells not viable
HL23–4	12/13/74	peripheral blood	pass 8	neg.	virums may be detected by direct cocultivation
HL23–5	12/13/74	bone marrow	pass 5	pass 5	virus = HL23V-5, transmitted to secondary cells (N. Teich and R. Weiss, personal communication)
HL23–6	01/25/75	peripheral blood	neg.	NT	cells grew poorly
HL23–7	01/25/75	bone marrow	neg	NT	cells grew poorly
HL23–8	01/25/75	bone marrow	NT	NT	used for cocultivation only; results neg.

a RT = reverse transcriptase, EM = electron miscroscopy, pass = passage number at a split ratio of 1 : 2, neg. = negative, NT = not tested.
b Cell specimens 1 and 2 were from one fresh blood sample. Specimen 1 was cultured immediately after receipt; specimen 2 was cultured beginning 10/01/74 from an ampoule of cells frozen on 10/16/73. Virus now isolated from passage 5, 7, and 10 of specimen 1. Original results *all* on pass. 10 isolate.

It is important to emphasize certain facts about these virus isolates in regards to the possibility that they might represent contaminaion with SSV. Our reasons for believing these isolates are not contaminants are as follows. (1) The fresh uncultured cells of the patient contained proteins (33, 48) and nucleic acid (38) related to the virus isolates as have several (but not all) other patients. (2) The same virus was isolated several times from different specimens from the same patient (see Table 3) after only short term passages in culture. A contaminant would have occurred with the same virus several times with the same patient and only this patient. (3) Other laboratories have independently isolated the same or a very related viruses from human tissues.

Table IV: Reported isolations of Virus of the »Woolly Monkey« (Simian) Sarcoma Virus (SSV)

Source of Isolate	Species and History	Reference
1. fibrosarcoma	woolly monkey pet of a woman	Wolfe, L., Deinhardt, F., Theilen, G., Kawakami, T., and Bustad, L., *J. Nat. Can. Inst.* 47:1115, 1971.
†2. acute myelogenous leukemia blood cells	Human. classical AML of a woman. Cells treated with growth factor	Gallagher, R. and Gallo, R., *Science.* 187:350, 1975.
†3. acute myelogenous leukemic bone marrow	Human. Same patient as #2. Cells treated with growth factor	Teich, N., Weiss, R., Salahuddin, Z., Gallagher, R., Gillespie, Gallo, R., *Nature.* 256:551, 1975. Gallagher, R., Salahuddin, Z., Hall, W., McCredie, K. and Gallo, R., *PNAS* (72:4137,
†4. normal lung fibroblasts	Human. embryo, 8 weeks gestation	Panem, S., Prochownik, E. V., Reale, F. R., and Kirsten, W. H., *Science.* 189:297, 1975.
5. normal lung fibroblasts	Human. embryo 16 weeks gestation	Panem, S. (as above)
†6. lung carcinoma cells co-cultivated with XC cells	Human. chronic lymphocytic leukemia and lung cancer	Gabelman, N., Waxman, S., Smith, W., Douglas, S. D., *Int. J. Cancer.* 16: 1, 1975.
* 7. lymphosarcoma leukemia bone marrow cells	Human. cells co-cultivated with XC cells	Nooter, K., Aarsen, A. M., Bentvelzen, P., de Groot, F. G., van Pelt, F. G., *Nature* 256:595, 1975.
* 8. lymphosarcoma leukemia blood cells	Human. cells co-cultivated with human embryonic fibroblasts	Nooter, K., (as above)
* 9. acute myelogenous blood cells	Human. Same patient as #2 and #3. Different sample	Markham, P. (unpublished)

* With these isolates data is not complete to say with certainty that virus belongs to "SSV family."
† In several instances with human isolates (those marked with symbol †) virus highly related to the baboon type-C virus was also present with the woolly monkey related virus.

Conclusions

We believe human myelogenous leukemia blood cells do not frequently permit *complete* expression of type-C viral information, but this information is at least

partially present in many and perhaps all AML patients. This is in contrast to the case of some animals like cats where most animals with leukemia actively produce virus. On the other hand, even with cats there is variation. The occasional (or rare) infected animal does not completely express virus (M. Essex, personal communication). Conversely, patient A. S. (HL-23) may be the unusual or rare human, who after appropriate growth stimulation of her leukemic cells, expresses completely and releases whole virus. One difficulty with our interpretation is our inability to detect the complete provirus.* This results in a paradox revolving around the question – how do human leukemic cells become transformed and how can they release virus if they lack the complete genetic information apparently essential in animal model systems for transformation and virus production? We think that generally the integrated complete provirus may be in only a small number of cells, perhaps not even the leukemic cell precursors. Release of fragments of the provirus by the infected cells may be sufficient, in some instances, to transform leukocyte precursors. This model is compatible with the existing data on human leukemia, including the detection of extra sequences in human leukemic DNA by Spiegelman and associates (41). At least one tissue or cell population should contain cells with complete provirus. *Portions* of this provirus may integrate into leukocyte progenitors, a necessary prelude to leukemic transformation. On occasion complete provirus may integrate into some leukocyte precursors, the necessary event for the rare complete virus production. We suggest that the site of integration for fragments or whole provirus is the "hot spot" region discussed before at this workshop (4) and that this may alter gene expression by a mechanism called "paraprocessing" (1) which in turn leads to transformation. If this speculation is correct, detection of the complete provirus as the proof for the involvement of these viruses in man will be extremely difficult.* Other approaches will be necessary such as additional virus isolates from other laboratories and/or a clear seroepidemiological studies.

* *Note added in proof*
Recently, we have for the first time been able to identify a DNA provirus in humans. We have found the provirus of a virus highly related or identical to the baboon endogenous type-C virus in the DNA of uncultured tissues from several but not all patients with leukemia (F. Wong-Staal, D. Gillespie, and R. Gallo, *Nature,* July 1976). We believe these results conclusively demonstrate that humans are infected by type-C virus. The results suggest an interspecies transfer of virus from baboon to man in the past. Whether the virus now spreads by way of an intermediary vector or directly – human to human is not known. There is no proof that this acquired viral information is causatively involved in leukemia although we naturally suspect that it may be. Since a major component of the repeated isolates of HL23 virus from a patient with AML is highly related to the baboon endogenous type-C virus, the new results clearly indicate that these isolates are from the patient not from laboratory contamination.

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