Yuri Ovchinnikov Memorial Lecture

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In memoriam Prof. Yuri Ovchinnikov

V. T. Ivanov

Who was Yuri Ovchinnikov? What were his ideas and what did he achieve during his dynamic although brief carrier? Why do we remember him at this Wilsede meeting? I do not think that these questions really ought to be answered, since Yuri Ovchinnikov belonged to the elite of international scientific community, being extremely well known not only for the results of his own research but also as an outstanding leader of chemical and biological sciences in the Soviet Union and a champion of international scientific collaboration.

A separate chapter of this book describes the path along which the main achievements of Ovchinnikov as a biochemist were reached. Here, I would like to offer the reader a glipse of Ovchinnikov as a human being. He was born in 1934 in Moscow; in 1952 he entered the Chemical Department of Moscow University and in 1957, after graduation, he became a professional researcher. Many facets of his unique personality showed up already in these early years. He had a phenomenal memory and was a leading actor at the University studio.

Yuri loved sports. He was a University champion in free-style wrestling, and was also a keen swimmer and cross-country skier. These skills he maintained for many years to come. It seems that the famous Robert Woodward has little

Fig. 1. Moments of relaxation (1970): Yuri Ovchinnikov (*left*) and Nobel prize winner Prof. R. Woodward (USA)

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Fig. 2. Yuri Ovchinnikov lecturing (1972)

Fig. 3. Deep in thought (1975)

chance of surviving in the billiard game with Yuri shown in Fig. 1.

Being a loving husband and father and generally a very warm personality, Ovchinnikov had a talent for collecting teams of young enthusiastic people and helping them in their research.

A clear vision, high motivation, the burden of responsibility – these are some

Fig. 4. At Yaroslavl airport (1982)

of the characteristic Yuri's qualities and feelings which are well reflected in Figs. 2, 3. A favorite among many of those who knew Yuri well is Fig. 4, where we see his firm stand, the boyish mischief, the invincible optimism and the belief that "we shall overcome", whatever the obstacles.

Indeed, he overcame much, but one thing proved too hard even for him: disease, the disease which could have been defeated if we knew more about the hemopoietic process. Maybe this is one more reason for dedicating the Volga Wilsede meeting to the memory of Yuri Ovchinnikov.

Prof. Yuri Ovchinnikov

V. T. Ivanov and V. A. Nesmeyanov[1]

Yuri A. Ovchinnikov started his carrier within the precincts of Moscow University at the Chemical Department under Professor Yu. A. Arbuzov. The project of his masters' degree (1957) provided material for the first publication on a new technique for the synthesis of pyrrolidine and thiophan derivatives. By that time, the gifted student had already shown a disposition toward synthetic organic chemistry. It was at this period that his belief took shape that the chemistry of living organisms was by far the most attractive area for an organic chemist to enter. Therefore, having begun his postgraduate course at the Chemical Department, Y. A. Ovchinnikov readily accepted an invitation to participate in the project on the complete synthesis of an important group of antibiotics, the tetracyclines. While working toward his doctorate, Yuri Ovchinnikov met M. M. Shemyakin, the leader of the project. The joint work led to a long-lasting collaboration between the two scientists, whose contribution to the foundation and advancement of physicochemical biology in the USSR was outstanding.

After finished his postgraduate course, Ovchinnikov joined the Institute for Chemistry of Natural Products of the USSR Academy of Sciences, set up not long ago. Here, Professor Shemyakin proposed that he go into peptide chemistry.

The subject under study depsipeptide antibiotics, atypical peptides containing hydroxy and amino acid residues. The problems of synthesis of the optically active N-methylated amino acids, reversible protection of the hydroxyl function of hydroxy acids, and cyclization of linear depsipeptides were rapidly solved and compounds with the structures proposed in the 1940s by Swiss researchers for antibiotics enniatins A and B were prepared. However, the samples obtained were devoid of antimicrobial activity and their physicochemical properties differed much from those of the naturally reliably confirmed, it remained to conclude that the formulae proposed for enniatins A and B were incorrect. Several alternative structures differing in ring size were suggested and accordingly synthesized. Two of them were indistinguishable from the natural enniatins A and B, which meant a solution to structural problems.

Later (1964–1970), Ovchinnikov and his colleagues performed a series of elegant syntheses of some other naturally occurring depsipeptides (sporidesmolides I-IV, angolide, serratamolide, esperin, beauvericin). he was awarded a D.Sc. in 1966 for the synthesis of natural depsipeptides and their analogs.

In 1967, Shemyakin, Ovchinnikov, and their team formulated the original (so-called topochemical) principle of transformation of biologically active peptides: novel molecules can be designed by such deep structural modifications as reversal of the acylation direction and the configuration of asymmetric centers, replacement of ester bonds by amide bonds and vica versa, cyclization of linear molecules,

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etc. The conditions favorable for retaining the original stereoelectronic parameters and, consequently, biological properties of the molecule were found. Ideas from this pioneer research were taken up by many laboratories and served to create novel highly active peptides (hormones, antibiotics, neuropeptides, enzyme substrates, and inhibitors).

The experience accumulated during this synthetic work served as a basis for the next and the culiminative step in studying the depsipeptide antibiotics. Bearing in mind the recently discovered ability of valinomycin and enniatins to induce permeability of lipid membranes to alkali metals ions, Y. A. Ovchinnikov and his colleagues undertook a study of the physicochemical basis of the phenomenon. It appeared that valinomycin binds potassium ions in solution, yielding stable complexes, and shows a unique K/Na-selectivity of complex formation unsurpassed in nature. Enniatins bind virtually all alkali and alkali-earth cations, though with a lower selectivity. These complexes are the ion-transporting species, and selectivity of ion binding is the origin of the selectivity of transmembrane ion transport. Further, the three-dimensional structures of the free antibiotics and their complexes were established. It was shown for the first time that such sophisticated structures can be resolved not only by X-ray analysis but also in solution by spectral methods. The bound ion appeared to reside always in the center of the depsipeptide molecular cavity and be kept in place by ion-dipole interactions with the carbonyl oxygens. The size of the valinomycin cavity is limited by a bracelet-like system of six intramolecular hydrogen bonds that accounts for its inability to adapt to smaller sized ions such as sodium or lithium. Enniatin structures are more flexible, which enables adjustment of the cavity to the size of the bound ion. The molecular periphery of both valinomycin and enniatin complexes is fully hydrophobic, which allows them to migrate freely across lipid zones of the membrane. Several laboratories outside the USSR were about to get similar results, but "the train had already gone."

Step-by-step protein compounds, the major working bodies of any living system, began to occupy the prominent place in Ovchinnikov's research activity. The 1970s witnessed a series of studies on the primary structures of porcine aspartate aminotransferase, and toxins from the venoms of cobra, bee, scorpion, etc. As a result, more than 20 structures were added to international data banks and atlases of protein structures. Inspired by these advances, Ovchinnikov and his group tackled the deciphering of the primary structure of *E. coli* DNA-dependent RNA polymerase, a key transcription enzyme investigated in many laboratories. Ovchinnikov had a very strong team, but even for them the problem seemed extremely difficult, since RNA polymerase is built of several subunits, among them two very large β- and β'-subunits (each over 1300 amino acid residues). Indeed, after rapid sequencing of the α-subunit (over 300 amino acids) it became clear that analysis of the β- and β'-subunits exclusively by conventional methods of protein chemistry could take many years. A decision was made to utilize the methods of genetic engineering and to analyze the sequences of genes coding for the subunits. In those days, such an approach was new for this country, and elsewhere it was at the early stages of development.

Genes for large subunits of DNA-dependent RNA polymerase form the so-called operon rpoBC and contain about 10000 base pairs. They were isolated, inserted into plasmids, and sequenced. Structures of peptides of large subunits were detected in parallel and independently. That was of use: when the structural analysis of genes was completed and the structures of corresponding proteins were derived according to the genetic code, they appeared to coincide with the peptide structures and, consequently, were determined correctly. Soon after that, other laboratories reported the gene

fragments but not the complete gene. It is worthwhile noting that the structures of these fragments contained errors. Only the combined use of the methods of protein and nucleotide chemistry provided reliable results.

The structural analysis of RNA polymerase served as a basis for a thorough investigation of the mechanism of action of the enzyme, for numerous genetic and biochemical studies.

That was in the late 1970s. More and more laboratories outside the USSR were successfully applying genetic engineering methods to microbiological synthesis of practically important proteins. Yuri Ovchinnikov was the first in the USSR to assess the prospects. He united enthusiasts and headed the work on improving the methods of chemical synthesis and directed mutagenesis of DNA to create microorganisms producing alien peptides and proteins. As a result, strains producing an opioid neuropeptide, leucine-enkephalin (1979), the antiviral and antitumour human protein interferon-α_2 (1981), and the precursor of human insulin, proinsulin (1983), were obtained.

Despite these advances of Yuri Ovchinnikov in genetic engineering and biotechnology, the bioorganic chemistry of peptides and proteins was always his major interest and devotion.

In the mid-1970s, he, N. Abdulaev, and a group of colleagues focused their interest on the molecular mechanisms of photoreception. By that time, a series of substantial discoveries had been made that paved the way for solving the problem of how light energy is transformed into the electric energy of the nerve impulse by rhodopsin, a well-known light-sensitive protein from the animal retina.

Soon after wards, there appeared data on the membrane protein – bacteriorhodopsin – found in microorganisms living in salt lakes. The protein was given that name because of its similarity to the visual rhodopsin (the presence of the bound retinal, light-sensitivity, etc.). Though bacteriorhodopsin functioned as a light-dependent proton pump, from the viewpoint of the primary photochemical properties it was very similar to rhodopsin. At the same time, bacteriorhodopsin is more readily available in large amounts and has a simpler structure than the visual rhodopsin, the main effort was initially directed to that protein. It was also considered that bacteriorhodopsin was (and still is) an ideal model for structure-functional analysis of membrane proteins. Simaltaneously with Prof. G. Khorana of the USA, the Nobel prize winner, Ovchinnikov succeeded in determining the amino acid sequence of bacteriorhodopsin, is was the first time that the chemical structure of the membrane protein had been deciphered (1987). Ovchinnikov and his team were then pioneers in solving the structure of rhodopsin from bovine retina (1981).

Research into the topography of polypeptide chains of these proteins in native membranes and elucidation of the structure of their active sites and disposition of functionally important groups were the next steps in this project. Using a variety of approaches including chemical modification, enzymatic treatment, and immunochemical methods, Yuri Ovchinnikov and his colleagues demonstrated that the two rhodopsins are arranged in the membrane in a similar way – as seven extended protein segments spanning the membrane's width and connected with each other on the two sides of the membrane by short peptide links.

In the mid-1980s, Y. Ovchinnikov and V. Lipkin focused their attention on the studies of other proteins involved in transmission and amplification of the visual cascade – transducin and cyclic GMD phosphodiesterase. In 1985, the primary structures of the γ- and α-subunits of transducin from bovine retinal rods were sequenced. Interestingly, the γ-subunit is characterized by the two adjoining cysteine residues also connected by a disulfide bridge. The residues are apparently involved in the formation of

the transducin-photoactivated rhodopsin complex.

An exciting page in the scientific biography of Yuri Ovchinnikov was his last project, devoted to studies of the system of active ion transport, i.e., Na,K-transporting adenosine triphosphatase and related proteins. In the late 1970s, Ovchinnikov initiated research into the structure of Na,K-ATPase. At the beginning, oligomeric organization of the functionally active complex in the native membrane was unraveled and the asymmetric arrangement of the subunits described. Further progress depended upon determination of the amino acid sequence of the subunits. Around 1985–1986, Ovchinnikov's team completed studies of the nucleotide sequences of genes for subunits and amino acid sequences of their polypeptide chains, which led to the complete primary structure of Na,K-ATPase from pig kidney outer medulla. Some research centers outside the USSR were also working intensively in these areas. The teams of S. Numa (Japan) and A. Schwartz (USA) simultaneously reported amino acid sequences of similar enzymes from other sources.

However, the approach chosen by Ovchinnikov extended far beyond the primary structure determination. Complemented by spectroscopic and molecular modelling studies, it resulted in the first detailed model of the Na,K-ATPase spatial structure. Here, the α-subunit (1016 amino acid residues) forms seven transmembrane segments and the major portion of its hydrophilic region accommodating the catalytic site is located inside the cell. The β-subunit (302 residues) spans the membrane once and the main part of its polypeptide chain forms an extracellular glycosylated domain.

As for the Na,K-ATPase active site, Ovchinnikov and his team employing affinity modification by ATP analog succeeded in identifying an unknown component of the catalytic site, thus experimentally confirming its dynamic changes during enzyme functioning.

Yuri Ovchinnikov, together with Eugene Sverdlov and their groups of researchers, obtained novel data on the regions of the human genome encoding the systems of active ion transport that seem to be of general biological significance. A family of at least five genes was defined in the human genome coding for several isoforms of the Na,K-ATPase catalytic subunit as well as other structurally similar ion-transporting ATPases.

The discovery of the multigene family gave rise to new concepts on regulation of the active ion transport through changes in the activity of the appropriate genes. This was supported by experiments on the expression level of various genes for Na,K-ATPase in healthy and pathological human tissues. Thus, ideas on the mechanisms of genetic regulation of ion-transporting enzymes received a solid foundation.

Lately, the problems of immunology and hematology attracted the attention of Yuri Ovchinnikov, who believed that chemistry and biology should do more to help solving medical problems in the USSR. Intense investigations of naturally occurring regulators of immunity and hemopoiesis have been started at the Shemyakin Institute. Some presentations at this symposium deal with these problems.

Above, we have outlined the scientific interests of Yuri Ovchinnikov, who was also in the driving seat in leading the chemical and biological scientific communities of his country. Ovchinnikov could not imagine how the science could evolve without intensive international cooperation. He excellently presented the advances of the Institute, and promoted scientific contacts, giving impetus to a series of bilateral symposia such as USSR–FRG, USSR–USA, France–USSR, Sweden–USSR, and Italy–USSR in various fields of physicochemical biology, many of which have now became a tradition. The remarkable symposia on Frontiers in Bioorganic Chemistry and Molecular Biology in Tashkent (1980) and Moscow-Alma-Ata (1984)

were also organized and presided over by him.

Of Yuri Ovchinnikov occupies a prominent place in the world's scientific heritage. We can only guess at what his further endeavors would have been, if he were still alive. It is our hope that this numerous works will inspire many generations of bioorganic chemists to come, providing the key to solving a diversity of problems and demonstrating again and again the beauty and the attractive power of the world of science.

Yuri Ovchinnikov Lecture

Human Leukemia Genes: Search for the Villains

J. D. Rowley

This Ovchinnikov Lecture provides an occasion to review our progress in a central area of cancer research, namely the genetic changes that occur within the cancer cell that are critically involved in the transformation of a normal to a malignant cell. To concentrate on genes to the exclusion of cell biology would be too narrow and shortsighted a perspective. Nonetheless, I am convinced that until we have isolated the genes that are centrally involved in at least some of the malignant processes in different cell types we will be unable to answer the fundamental questions about malignant transformation. More importantly, we will be unable to answer the questions with precision. I will limit my consideration to those changes that have been detected by analyzing the karyotypic pattern of human cancer cells using chromosome banding, and in particular to those found in leukemia.

We are living in a golden age of the biomedical sciences. Increasingly sophisticated instruments and creative scientific strategies allow remarkably precise understanding of some aspects of cancer biology. It is clear that during the course of the last three decades, the scientific community's assessment of the role of chromosome changes in the complex process of malignant transformation has changed from considering them to be merely trivial epiphenomena to recognizing their fundamental involvement at least for some tumors. This change in attitude has occurred for at least two reasons. First, the demonstration of specific recurring chromosome rearrangements, including translocations and deletions, that were often uniquely associated with a particular type of leukemia, lymphoma, or sarcoma provided clear evidence that these rearrangements *were* critically involved in malignant transformation [1–3]. About 70 recurring translocations as well as many nonrandom deletions and other structural abnormalities are listed in the chapter on structural chromosome changes in neoplasia included in *Human Gene Mapping 10* [4]. The evidence for the presence of recurring chromosome abnormalities in a wide variety of human neoplasms was the result of 30 years of painstaking chromosome analysis by my cytogenetic colleagues around the world.

Second, and I believe an even more powerful force acting within the general scientific community to reassess the role of karyotypic alterations, was the identification of the genes involved in some of the chromosome rearrangements and the discovery that some of these genes were the human counterparts of the viral oncogenes [5]. In a sense, each group of investigators gave the other scientific validity. The fact that oncogenes were directly involved in chromosome translocations demonstrated that *both* translocations *and* oncogenes were critically involved in human cancer.

The genetic changes that occur in different types of malignant cells are quite varied, and clearly several different changes occur in the same cell as it is altered from a normal to a fully malignant cell. Cytogenetic analysis has been the key to defining at least two major categories of rearrangements, namely recurring translocations and consistent de-

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letions. One of the first translocations, identified in 1972, was the 9; 22 translocation in chronic myeloid leukemia [6]. There are now at least 70 recurring translocations that have been detected in human malignant cells. The identification of consistent chromosome deletions has been equally important because it has provided the absolutely essential information regarding the chromosome locations of the genes that are involved in cancer. I submit that the retinoblastoma gene would not have been cloned, or at least not yet, if cytogeneticists had not identified deletions of the long arm of chromosome 13, and specifically of band 13q14, in patients with constitutional chromosome abnormalities who had a high incidence of retinoblastoma [7]. This is not to detract from the careful and exciting work of many scientists is actually cloning the gene, but at least they knew where to look [8]. This triumph has now been joined by the recent cloning of the *DCC* (deleted in colorectal carcinomas) gene on chromosome 18; the fact that a gene important in the transformation of colorectal cells was located on chromosome 18 was the result of cytogenetic analysis of colon cancer cells that revealed that loss of chromosome 18 was a recurring abnormality [9–10].

I must acknowledge that it has been a source of great disapointment to me that we have progressed so slowly in cloning most of the genes located at the breakpoints in the recurring translocations or inversions in human leukemia. This emphasizes the fact that knowing the location of the breakpoint is very helpful in selecting the genes to use as probes for these rearrangements. However, a chromosome band contains at least five million base pairs and the likelihood that the DNA probe that you "pull off the shelf" is at the breakpoint and can detect a rearrangement on Southern blot analysis is vanishingly small. The lymphoid leukemias and lymphomas are the major exceptions to this slow progress, because the immunoglobulin genes in B cell tumors and the T cell receptor genes in T cell tumors have provided the essential DNA probes to clone several dozen translocations [11–13].

Fortunately, the rapid progress being made in mapping the human genome, coupled with major advances in working effectively with large pieces of DNA, has already made important contributions to the successful mapping of some of the recurring translocations in the acute leukemias and sarcomas. The use of cosmids or yeast artificial chromosomes (YAC) as probes to screen much larger segments of DNA for rearrangements provides a new strategy for the analysis of these chromosome abnormalities. We have used these probes in in situ hybridization with biotin labeling of the DNA and detection with a fluorescein isothiocyanate-(FITC)-tagged avidin–antiavidin conjugate. The focus of our research has been the analysis of chromosome translocations involving band 11q23. This band is of great interest because it is affected in a large number of different recurring rearrangements. The translocations may occur in either acute lymphoblastic or acute myeloid leukemias, especially of the monoblastic or myelomonocytic subtype. Finally, about two-thirds of chromosome abnormalities in leukemia cells of children under 1 year of age involve 11q23, regardless of the morphological classification of the leukemia.

We have used a series of cosmid probes as well as a yeast clone containing two YACs to map the 11q23 breakpoint in four different translocations, namely t(4;11), t(6;11), t(9;11), and t(11;19) [14]. The cosmid probes were isolated by Evans et al. [15] and they were mapped to the region 11q22 to 11q25 by Lichter et al. [16]. The yeast clone with the YACs was identified using polymerase chain reaction primers specific for the *CD3G* gene. We showed that this yeast clone contains two YACs of 320 and 275 kb that differ only because of a 45 kb deletion in one of the YACs; the deletion is in the end opposite the *CD3G*/*CD3G* complex. With the use of cosmid probes we

obtained essentially the same results in all four translocations. The cosmid probes 3.16, 23.20, 1.16, 4.13, ZB6, and *CD3D* all remained on chromosome 11. The cosmid probes XH5, XB1, ZC9, *PBGD*, 9.4, ZA7, *THY1*, 8.5, *SRPR*, XB2, *ETS1* 23.2, and 5.8 all were translocated to the other chromosome. Seven cosmid probes (XH5, XB1, ZC9, *PBGD*, 9.4, ZA7, and *THY1*) were deleted in one t(9:11) patient, presumably simultaneously with the translocation.

The *CD3G* YACs localized only to chromosome 11 in normal cells. However, in addition to labeling the normal chromosome 11, the YACs were split in cells with the four translocations; thus one portion remained on chromosome 11 and the other was translocated to the other chromosome. There was no labeling of any other chromosomes in these cells. Thus the breakpoint in these translocations, which occur in both lymphoblastic and myeloid leukemia, is within the same 320 kb region of human DNA. We have no evidence, at present, of whether the break involves the same segment in the different translocations. Thus the use of YAC clones provides a new strategy for screening large pieces of DNA and for focusing intensive molecular analysis only on the segment that is shown cytogenetically to be of interest. YACs will also be of great benefit in defining the genetic boundaries of chromosome deletions. These probes also provide powerful tools for detecting these same rearrangements in interphase cells.

Using more conventional techniques, a colleague of mine, Dr. Timothy McKeithan, has cloned the translocation breakpoint found in some patients with B cell chronic lymphatic leukemia [17]. The translocation involves the immunoglobulin heavy chain locus (*IGH*) located at chromosome band 14q32 and a previously unknown gene that we have called *BCL3* on chromosome 19 (band 19q13). They cloned the translocation breakpoint from two of our patients as well as from several others from material provided by other laboratories. There was evidence for rearrangement adjacent to one of the *IGH* constant regions in each case. In four of the cases, this rearranged band has been cloned; all showed a rearrangement with sequences from chromosome 19. Three of the breakpoints on chromosome 19 were within 170 bp of each other; the fourth lay 19 kb centromeric. Overall, a region of about 35 kb surrounding these breakpoints has been cloned and mapped.

A cluster of CG-containing restriction sites was found close to the cluster of breakpoints on chromosome 19. These "CpG islands" are usually associated with the 5′ ends of genes. The presence of a CpG island adjacent to the cluster of t(14;19) breakpoints was confirmed by sequencing. Probes from this region were used in Northern blot experiments, which detected a 2.1–2.3 kb transcript in many hematopoietic cell lines. S1 protection experiments confirmed this result and showed that transcription occurred in a direction away from the breakpoint toward the telomere.

The *BCL3* cDNA was cloned and sequenced [18]. A basic protein of 446 amino acids and a molecular weight of 46741 is predicted, which shows a remarkable structure. The N-terminus is highly enriched in proline (25%), and the C-terminus in proline (23%) and serine (28%). Almost the entire remainder of the protein (about half) is made up of seven tandem repeats of 33–37 amino acids. Comparison with proteins in the available data bases showed significant homology to the *Drosophila* Notch protein. The homology is in the region of the repeats. Notch has six repeats with clear similarity to the repeat in BCL3. These repeats have been found in three additional proteins – namely, lin-12 of *Caenorhabditis elegans* (six repeats), cdc10 in *Schizosaccharomyces pombe* (two) and SW16 in *Saccharomyces cerevisiae* (two) [reviewed in 18]; the repeats are generally referred to in the literature as cdc10 repeats. The role of this motif is not known.

Total RNA from two patients with chronic lymphocytic leukemia (CLL) and the t(14;19) – one with a break on chromosome 19 close to *BCL3* and one with a break more than 25 kb away – was hybridized on Northern blots to determine the level of *BCL3* expression in cells containing the t(14;19). The samples were compared with total RNA from the peripheral blood of two other patients with CLL, as well as with three cell lines derived from the prolymphocytic variant of CLL; none of the cell lines contain the t(4;19). In addition, five other hematopoietic cell lines were examined. The level of message in the two CLL samples with a t(14;19) was higher than that found in any other sample examined. By hybridization to blots containing various quantities of RNA, the two t(14;19) samples were found to contain 5–7 times and 10–15 times the level of message present in the CLL cell line with the greatest quantity of message [18]. The message present in the cells with the t(14;19) was identical in size to that present in normal hematopoietic cells, as would be expected from the fact that the translocation breakpoints occur upstream of the transcription start site. The apparent normality of the message suggests that the increased message level results from increased transcription and not from an increased message stability arising from changes in the structure of the transcript itself.

The known functions of the other proteins containing the cdc10 motif may offer a clue to the function of BCL3; unfortunately, however, the divergent structure and function of these proteins makes it difficult to image a common role for the motif. Notch (in *Drosophila*) and lin-12 (in the nematode *Caenorhabditis*) are transmembrane proteins involved in cell lineage determination. On the other hand, the two yeast proteins are not transmembrane proteins and they share functions involved in control of the cell cycle. *cdc10* is one of two genes in *Schizosaccharomyces pombe* known to be required for commitment to the cell cycle; this control point, in G1, is known as "start". Much more is known about the function of the other required gene, *cdc2*, encoding a protein kinase which is highly conserved among eukaryotes and is required both for start and for mitosis. Little is known about the function of the cdc10 protein. Recently, a specific antibody to cdc10 was shown to detect a protein of similar size in mammalian cells, suggesting that, like cdc2, the protein may be conserved throughout the eukaryotes.

SW16 is one of several genes known to be required for transcription of the *HO* gene, which encodes the endonuclease which initiates mating type switching in *Saccharomyces cerevisiae*. *HO* is activated immediately after commitment to start, and a particular repeated sequence in the 5′ flanking region of the gene has been shown to be responsible for cell cycle control of its transcription. *SW16* and *SW14* (whose sequence has not yet been reported) are the only genes known to be specific for this control element. These two genes appear to be at least partially interchangeable since neither single mutation is lethal, but double mutations are nonviable. While the function of *SW16* strongly suggests that it is a nuclear *trans*-activating protein, it has not been directly shown to interact with DNA or even to be a nuclear protein.

If the cdc10 motif is involved in protein-protein interactions, there may be little commonality in function between the two yeast proteins and the two invertebrate proteins. Nevertheless, there are a few plausible models in which the proteins could have related functions. For example, BCL3, cdc10, and SW16 may be peripheral membrane proteins which interact with the cytoplasmic domains of transmembrane proteins and are involved in signal transduction. According to this interpretation, the ancestor to the *lin12* and *Notch* genes could have resulted from the fusion of two genes in evolution – one encoding a transmembrane protein, and the other, a cdc10-related protein.

The increased levels of *BCL3* message following mitogenic stimulation and the homology of the gene to cell cycle control genes suggest that abnormally large quantities of the protein present in CLLs with the t(14;19) may lead to an increased proliferative rate in these cells. This superficially seems inconsistent with the very low mitotic rate of CLL cells. Perhaps this mitotic rate, while low, is nevertheless greater than that of normal CD5+ B lymphocytes. Alternatively, a subpopulation of CLL cells, perhaps those present in pseudofollicular growth centers in lymph nodes, may show an abnormally high rate of proliferation.

One of the major reasons to concentrate on cloning the genes involved in rearrangements is that the consistent chromosome changes pinpoint the location of the genes whose functions are critical in the growth potential of that cell type. The chromosome changes that we concentrate on are present in all of the malignant cells; thus they are not random events affecting one or a few cells in the involved tissue. Moreover, they are clonal in origin and are derived from a single cell in which the intial chromosome change occurred. These changes are somatic mutations in individuals who otherwise virtually always have a normal kyrotype in their uninvolved cells. These observations provide the evidence that cancer is a *genetic disease*. This notion seems self-evident today, but it was not generally accepted several decades ago when many of us began working in cytogenetics. Clearly, I am using "genetic" in a special way, referring to changes in genes within the affected cell, not in the more usual sense of a constitutional genetic disease such as hemophilia or color blindness.

I will conclude with some comments regarding the longer-term potential impact of discovering new genes via chromosome rearrangements. Once these genes are identified, many previously unknown, *BCR* or *BCL3* for example, they become the focus of very active investigation. Scientists try to find answers regarding the function of these genes in normal cells; how are they altered by the chromosome rearrangements, and how does this relate to malignant transformation? The questions are endless. The answers will provide insights into cell biology that have very profound implications. Within the next decade or two, we should be able to define the major genetic abnormalities in many types of cancer and to identify the specific changes in the tumor cells of many patients. For most leukemias, lymphomas, and sarcomas, unique chromosome changes are often associated with a particular subtype of these neoplasms.

Cloning of the genes involved in these chromosome changes will provide specific DNA markers that will have diagnostic importance. For some solid tumors, on the other hand, current evidence suggests that deletions of the same chromosome region may occur in different types of tumors, such as the deletions of 13q in retinoblastoma, osteosarcoma (not secondary to radiation for retinoblastoma), breast cancer, and lung cancer. The deletion of the same region does not necessarily imply that the same gene is involved or that the change within the gene is identical, witness the fact that two different translocations in band 22q11 involve different genes, namely, the lambda light chain gene in the 8;22 translocation in Burkitt's lymphoma and the *BCR* gene in chronic myelocytic leukemia (CML); furthermore, the breakpoints within *BCR* in Ph^1-positive leukemia are also somewhat variable.

The multistep process of malignant transformation is complex. In the leukemias and lymphomas, we often see specific chromosome translocations combined with loss or gain of particular chromosome segments. Some combination of alterations in dominantly acting proto-oncogenes and in recessively acting tumor-suppressor genes certainly act synergistically to enhance the malignant phenotype.

In the future, the precise definition of the genetic changes in the malignant cells

of a patient will be used to select the most appropriate treatment for cells with these genetic defects. This treatment will be less toxic for the normal cells in the patient. Moreover, this genetic profile may allow monitoring of the patient's course and early detection of relapse. These same genetic markers may be used to detect the involvement of other tissues such as bone marrow, spleen, or lymph nodes. These changes in treatment strategies will clearly benefit the patient. Of more general scientific importance, however, will be the identification of dozens of genes, many hitherto unknown, that can be used to study the complex process of the regulation of cell growth and differentiation. This development may be the most significant result of our success in understanding the genetic changes that occur in cancer cells.

I would like to conclude with a more personal note based on my continuing amazement at the interrelatedness of the biomedical sciences. This should be no surprise to me, but it is. Many investigators have found that a successful system for carrying out some function in primitive organisms has evolved and then this system is used repeatedly with varying modifications as the organisms become more complex. As a cytogeneticist, I had to learn something about the cell cycle and DNA replication, about chromosome structure, and about various agents that can alter both of these. More recently, I have had to become an amateur tumor virologist at least with regard to the action of viral oncogenes and their cellular counterparts, the proto-oncogenes. With the cloning of translocations, especially some of the recent ones, a knowledgeable cancer cytogeneticist must understand cell cycle control genes in yeast (*cdc10* and *SWI4* and *6*) and developmentally regulated genes in *Drosophila* (*Notch* for example) and nematodes (*lin-10*, *glp-1*). I have already described in some detail, the cloning of the *BCL3* gene by McKeithan et al. [17, 18]. If I am to understand the possible roles this gene plays in B cell transformation, I must understand how its homology to portions of the *cdc10* and *Notch* genes might provide clues as to its functions in both normal and malignant cells. As more genes involved in translocations and deletions are defined, many of us in cancer research will continue to have to "go back to school" to be able to incorporate the knowledge provided by molecular geneticists and cell biologists into our concepts of carcinogenesis. The golden age in biology and in medicine are nourishing one another as never before in history.

Acknowledgements. My colleagues, Drs. Manuel Diaz, Michelle Le Beau, and Timothy McKeithan, have been generous with their review of this manuscript. I acknowledge the expert secretarial assistance of Ms Felecia Stokes. The research described has been supported by the Department of Energy Contract No. DE-FG02-86ER60408 and by United States Public Health Service Grant CA 42557.

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Yuri Ovchinnikov Lecture

Human Retroviruses: Linkage to Leukemia and AIDS

R. C. Gallo [1] and L. S. Nerurkar [1]

Introduction and Background

This review will discuss interactions of retroviruses with the cells of the hematopoietic system. Such interactions have been studied in the past as tools to insert genes in the cells to study their regulation or to study cellular and molecular basis of transformation in vitro. The emphasis of this review will be on viruses which cause diseases, particularly in man.

A decade ago there was no general acceptance of the concept that genes were critical to leukemias and lymphomas or to disorders of hematopoietic cells. In a somewhat analogous way there was also a general feeling that viruses did not cause human cancers, and that retroviruses, in particular, did not exist in human beings. We now know that viruses, either directly or indirectly, either as a cofactor or as a direct cause, play a role in more than 40% of human cancers. We have also learned that human retroviruses do exist and in multiple types.

During the 1970s, there was also a feeling in the United States that serious or fatal, epidemic or pandemic diseases were things of the past. Infectious diseases that would become global epidemics were no longer a problem for the so-called "industrialized nations." Such diseases were really a problem for the less-privileged nations. We had preventive and curative measures like vaccines and antibiotics in addition to better sanitary conditions and public health measures. In retrospect, we should have remembered that the last great pandemic that affected the United States, Europe and the world was only about 70 years ago. It was the great influenza epidemic of 1918–1920. And if one reviews the history of microbiology, there were often periods where epidemics disappeared and mysteriously reappeared after more than 60 or 70 years, or even for 100 or 200 years. Perhaps we were overconfident in thinking that epidemics belonged to the past: an epidemic or pandemic of the acquired immunodeficiency syndrome (AIDS) as now been with us for a decade. There was also a feeling that pandemic diseases were not possible unless the causative agents or the microbes were casually transmissible. We now know that we have a pandemic of AIDS and the agent is not casually transmissible, but transmissible only by close contact and with the exchange of body fluids.

The failure to appreciate the coming of these events was probably because of the failure to remember some of the lessons of past medical history; that often there are major changes in diseases following some major changes in the society. The major changes in the post-World War II era were: a great increase in air travel; the use of blood and blood products, often going from one nation to another; the insane habit of intravenous drug abuse; and the increase in sexual contacts. All these things made it possible to transmit something that was remote or rare so that it become relatively common and global. Comparing the epidemics of the past, the AIDS epidemic is not particularly novel,

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nor is the response to the epidemic by the public as is often portrayed by the media. The only novel feature of this epidemic is the nature of the microbes that are causing the epidemic. The novel properties of these microbes are: they are newly discovered (but are not new); they are microbes that are often difficult to find because they do not replicate much or do not infect many target cells; they are only produced by the infected cells, primarily during the proliferating phase of the cell's life cycle. In fact, this is true for all human retroviruses.

For the same reasons that they are difficult to find, the viruses are difficult to transmit. Almost always, they have a very long latency period. This is an important characteristic and has allowed their transmission to become global as they are present in the host from the time of infection until death (lifelong infection) and during that period can be transmitted to others. This is a major difference between retroviruses and other viruses, which we tend to think are transmitted while a person is sick or in the early phase of incubation, which can be a few days, a few weeks, or at the most a few months. The very long latency period of retroviruses means that it may be several years or several decades from the time of infection before the first manifestation of disease will be noted. They often cause serious diseases, e.g., central nervous system disease, malignancies, and immune deficiency. These agents have thus become increasingly important because of the serious and often fatal consequence of their infection.

Discovery of Human Retroviruses

There are four human retroviruses well characterized by now [1–3]. Human T-lymphotropic (leukemia) virus type I (HTLV-I) was found by Gallo and coworkers in the late 1970s and first reported in 1980. Its relative, HTLV-II, was also found in our laboratory a year or two later. The human immunodeficiency virus type 1 (HIV-1) or AIDS virus causing the epidemic we now face was found in 1983 by Barre-Sinoussi et al. at the Institute Pasteur [4] and established by our laboratory as the cause of AIDS in early 1984 with many isolations of the virus and the development of the blood test. The related virus from West Africa, called HIV-2, is neither as pathogenic as HIV-1 nor is it spreading like HIV-1. It appears to be almost limited to West Africa.

The technology developed in the 1970s, particularly, the sensitive assays for reverse transcriptase (RT), was crucial for the discovery of human retroviruses. The discovery of RT, by Temin [5] and independently by Baltimore [6], was quickly extended by the finding of a similar enzyme in human leukemic cells from unusual cases by Gallo and his colleagues during the 1970s [7]. Enzymes from at least four or five patients which had the properties of the viral enzyme were partially purified. More important was the development of synthetic template primers, e.g., synthetic polymers (oligo-dT-poly-A and oligo-dG-poly-C) that made the assays for these enzymes specific and sensitive. This improved the detection of retroviruses several-fold compared to the electron microscopic method used for decades. Also, the assay using RT is much simpler and cheaper and can be done continuously while the culture is ongoing. Electron microscopy does not offer that possibility. Retroviruses, including those affecting humans, complete their replication cycle much more efficiently during the proliferating phase of that cell's life cycle. RT assays performed continually on the cells in culture can reveal short-term viral replication which otherwise may be missed by electron microscopic techniques.

The second important technology was the ability to grow human T cells, particularly with interleukin-2 (IL-2), discovered by Morgan, Ruscetti and Gallo in 1976 [8]. Developments in the field of immunology such as monoclonal antibodies have allowed defining subsets of

lymphocytes by surface markers or by other assays to understand different functions of T-cell subtypes. A third factor contributing to the discovery of human retroviruses is the fact that they spread globally in the 1960s and the 1970s, and became much more common. We believe that this may have increased the chances of detecting and isolating them considerably. And the last point which is worth mentioning was the perseverance in looking for them, even though most scientists did not think they existed.

Classification of Human Retroviruses

Human retroviruses belong to two entirely different subclasses which differ in their morphology, some aspects of their genomic organization, and some aspects of their biology. The HTLVs belong to the more classic type of animal retroviruses known in most species as type C or oncorna retroviruses, whereas the HIVs belong to the category known as lenti-retroviruses. "Lenti" is not an accurate term, as it means slow. HIV does not replicate slowly compared to HTLVs. HTLVs are much more slowly replicating viruses, and thus the class names can be misnomers. Until the discovery of HIVs, lenti-retroviruses were only known to occur in ungulates, the hoofed animals like horses, sheep, cows, and goats [9]. One has to be careful in not drawing too much of an analogy between HIV and these ungulate lenti-retroviruses. However, there are some common characteristics, e.g., they infect cells of the macrophage lineage and morphologically their core structures appear similar. But there are major differences in other aspects. For example, some of the ungulate lenti-retroviruses can be transmitted casually. The visna virus of sheep is thought to be transmitted by fomites in crowded sheep that are herded together in a closed environment. None of the ungulate lenti-retroviruses target $CD4^+$ T lymphocytes and they are not known to be associated with the increased frequency of the development of a malignancy. More recently, we have other animal models, particularly the simian models, in which lenti-retroviruses have been isolated that are closer to the humans [10].

Morphology. The size and shape of HTLVs and HIVs are roughly the same. However, the core structure of the leukemia viruses is much different from that of the AIDS virus. The latter is much more condensed and cylindrical in shape compared to that of the leukemia viruses (see Fig. 1).

Biological Properties. The HTLVs and HIVs show many parallels in their biological characteristics. Both viruses infect $CD4^+$ T lymphocytes but they vary in the consequence of infection. The overall effect depends on the extent of virus replication and on the functions of some of the genes the virus carries. HIV-1 infection kills the $CD4^+$ T cells. HIV-2 essentially behaves in a similar manner. On the other hand, HTLV-I and HTLV-II, like most animal retroviruses, have no lytic activity on their target cells, but can alter the function of that cell. Some infected T cells become immortalized in vitro and may contribute in the same manner to the development of leukemia in vivo. Both classes of viruses remain latent in the patient or in the cell for their lifetime. Another feature both HIVs and HTLVs have in common is the tight control of the DNA-integrated provirus. Following the infection of a $CD4^+$ T cell by these viruses there is integration of their DNA forms into the host chromosomes, but the DNA forms do not induce expression of RNA or proteins. So an infected cell will have no viral RNA or viral protein immediately after infection. This means that the immune system cannot find the infected cells. This is one way these viruses escape the immune system. Other mechanisms which allow these viruses to escape are, for example, by infecting the brain and by undergoing considerable genomic variation from isolate to isolate (particularly in the case of HIV).

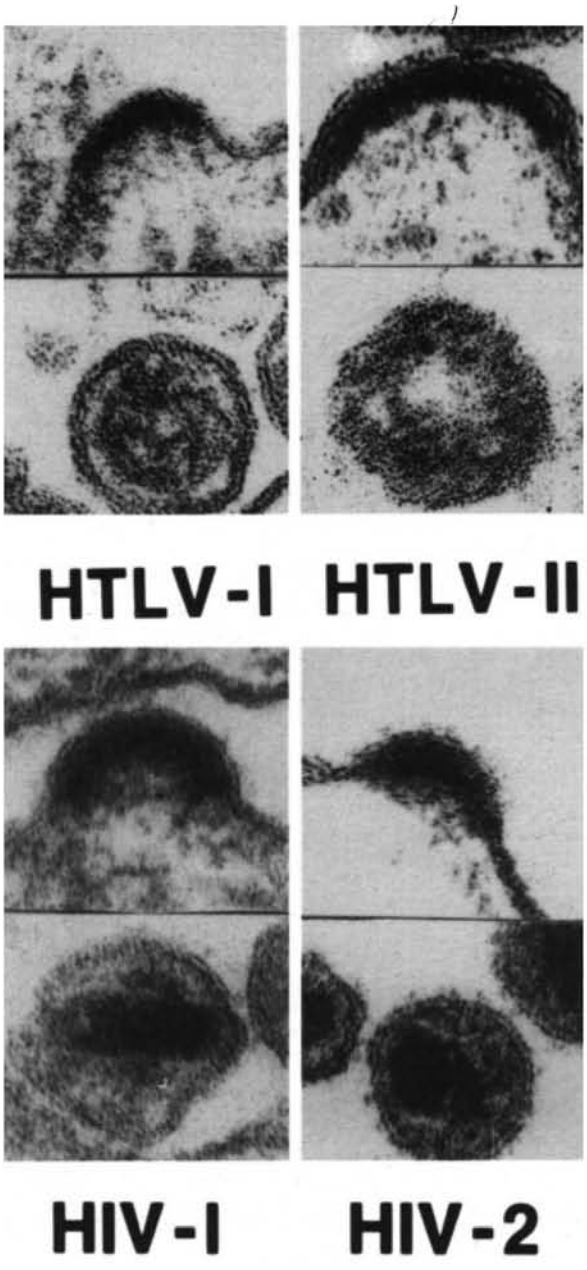

Fig. 1. Morphological structures of HTLV-I, HTLV-II, HIV-1, and HIV-2. The *top panels* represent the budding from the cell membranes, the *bottom panels* show the cross section of the mature virons

In addition, HIV destroys the cells of the immune system which are crucial in the immune surveillance itself, thereby escaping the immune attack.

When the T cells are immune-stimulated, perhaps by another infection, the viral genes become active along with a variety of other cellular genes and viral proteins are expressed on the cell surface. This allows the immune system to see the infected cell. Such immune clearance may be too late. The virus released from such cells infects other cells. In this manner, the HIV-infected host who has other chronic infections is more likely to spread this virus.

Modes of Transmission. The HIVs and the HTLVs have common modes of transmission. They are transmitted by blood or sex and from mother to child. For HTLV-I, males chiefly get infected from their mothers, and women chiefly get infected from their male sexual contacts. The mode of transmission from mother to child is *in utero*, transplacentaly as well as by milk or in the actual birth process. Blood transfusions and the use of blood products are, of course, also modes of transmission of both viruses, the major target cell being the $CD4^+$ T cell [11].

Analogous Animal Retroviruses. The closest relative of the human leukemia viruses, called simian T-cell leukemia/lymphoma viruses, are found in African monkeys [12, 13]. They are not found at all in New World monkeys, i.e., in monkeys from the North American continent and those from Asia [14]. But the viruses in African monkeys are closer to the human viruses than to those from Asian monkeys. Similarly, the closest relative of HIVs are also found in African monkeys. No relatives of HIV have been found in New World or Asian monkeys. Because of the fact that the closest relatives of all human retroviruses are present in African primates, the ancestral origin of these viruses is almost certainly African. That does not mean that the recent epidemic of AIDS came from Africa. As far as one can tell, the epidemic of AIDS began almost simultaneously in parts of Central Africa, some of the Caribbean islands, particularly Haiti, and the United States, and perhaps in Europe.

Geographical Distribution of HTLV-I

HTLV-I transmission is extremely tightly controlled and if one did not have a

handle on the virus (virus isolation or virus detection using probes), the diseases it causes, e.g., leukemia or neurological diseases, could be mistakenly thought to be genetically inherited. HTLV-I is endemic in Subsaharan Africa. It is not present in all parts of Subsaharan Africa, but seems to be restricted to certain tribes or geographical areas and is not casually transmitted. HTLV-I is also endemic in the Caribbean basin, including the northern part of South America, Central America, most of the Caribbean islands, and parts of the southeastern United States. Some Caribbean islands do not have any HTLV-I. It depends on where in Africa the island inhabitants have their origin. If the ancestral tribe is positive, then the descendants in some Caribbean islands are positive. Similarly, if the ancestral tribe is negative, then the descendants in another Caribbean island are negative for the most part. HTLV-I is also endemic in the southern islands of Japan in Shikoku, Kyushu, Okinawa, and other neighboring islands. Seroepidemiologic studies have suggested that clusters of HTLV-I, or a virus like it, are observed in some villages in Spain and in southeastern Italy in a region called Apulia. Manzari of Rome and Varnier of Genoa believe that the virus in Apulia in southeast Italy is endemic. It is not a coastal introduction from outside in recent times; rather, it is found in the people living in the interior hills. Recent molecular analysis studies of some of the isolates from that region indicate that it is not the classic HTLV-I, but may be another retrovirus related to HTLV-I. There have been clusters of HTLV-I-related leukemia reported in Amsterdam and London in migrating populations from the Caribbean. The rate of developing leukemia after HTLV-I infection is identical in populations which have migrated and in the nonmigrating population, indicating that no other environmental factor is needed for the cause of leukemia, at least as far as the epidemiology can determine [11]. For a great part of the world, we have very little data. For example, we do not know very much about infection by HTLV-I in the Soviet Union.

Nature of the Diseases Caused by HTLV-I

Leukemia. The picture of the first patient from whom a retrovirus was isolated is given in Fig. 2. This patient was a young black male and came from the southeastern part of the United States. He had no interesting past history, either medical, familial, or occupational. He developed a severe acute T-cell malignancy of the $CD4^+$ T lymphocytes. The skin manifestation in this disease is due to infiltrates of leukemic cells in the skin, which is a common feature in this disease [15, 16]. Frequently, there is high blood calcium, which can lead to death of the individual. Liberation of some lymphokines is suggested as a possible molecular mechanism for high blood calcium [17]. There is also an increased incidence of opportunistic infections and slight immune impairment can be observed in infected people. However, when a disease begins to develop, the course is very rapid. It resembles the chronic myelogenous leukemia going into blast crisis. Death usually follows in less than 6 months.

These manifestations of the disease are common, occurring in about 70%–80% of people who have leukemia with HTLV-I. Another 20%–30% show a more chronic course, and the diagnosis is of chronic lymphocytic leukemia of a $CD4^+$ T-cell type, mixed cell lymphoma, or histiocytic lymphoma of a $CD4^+$ T-cell type. So, in any $CD4^+$ T-cell malignancy, one has to consider the possibility of HTLV-I, and particularly if the disease is as aggressive as described above.

Neurologic Disease. It is now known that HTLV-I also causes a serious and fatal progressive neurologic disease which is similar to multiple sclerosis but can be distinguished from it. There is some confusion in this area because some labo-

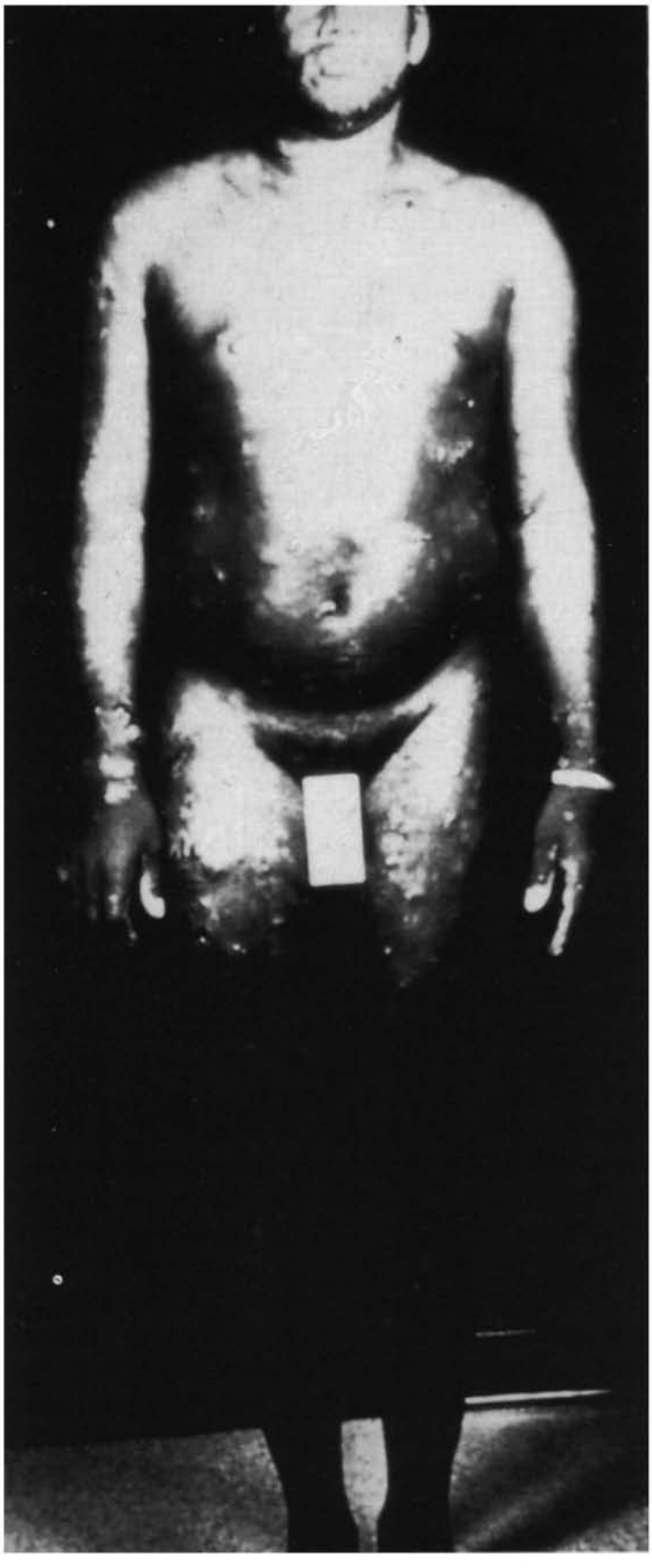

Fig. 2. The first patient with T-cell leukemia caused by HTLV-I

ratories have reported HTLV-I or a closely related virus as being involved in multiple sclerosis itself. The data are not consistent from laboratory to laboratory. More evidence is required to implicate HTLV-I or a relative of HTLV-I in playing a definitive role in multiple sclerosis. However, the neurologic disease that has been called tropical spastic paraparesis or Jamaican neuropathy, and sometimes misdiagnosed as multiple sclerosis or a variant of multiple sclerosis, is certainly linked to HTLV-I [18], although the disease mechanism is not understood. The HTLV-I-associated disease differs from multiple sclerosis in that it does not have exacerbations and remissions like multiple sclerosis: it is progressive. It is characterized by incontinence of the bladder, impotency in males, loss of bowel function and spasticity of the lower extremities. The disease can occur rapidly after infection with HTLV-I. It appears to depend on the dose of the virus.

There is a recent report of a Frenchman who received a transfusion with HTLV-I-positive blood, developed the neurologic disease in 5 weeks, and transmitted the virus to his wife during that period. This implies that all of the blood supply should be tested for HTLV-I as well as for HIV [19]. However, the neurologic disease could take many years to develop and there is some indication that genetic factors are important. There are some reports from Japan showing an HLA class 2 association and that certain patterns have an increased frequency of developing the neurologic disease. A known fact is that the virus is not found in the central nervous system tissues, e.g., brain cells or cells of the spinal cord, but only in the cerebrospinal fluid.

The other known facts are that people who develop the neurologic disease have a very high titer of antibody, much higher than the healthy carriers or the leukemic patients. Even more interesting are the recent results of Jacobson, McFarlin, and their coworkers, who describe high levels of cytotoxic T lymphocytes reactive against *tax* and *env* gene products [20]. This has led to the speculation that the immune response to the virus produces an autoimmune disease. Recent reports indicate that HTLV-I is also involved or linked epidemiologically to other diseases listed in Table 1.

Table 1. Diseases caused by or associated with human retroviruses

1. Adult T-cell leukemia (ATL)
2. Occasional other T4 leukemias/ lymphomas
3. Tropical spastic paraparesis (TSP) or HTLV-associated myeloneuropathy (HAM)
4. Mild immune impairment
5. Polymyositis
6. Rheumatoid arthritis-like disease (?)
7. Retinitis (?)
8. B-cell lymphocytic leukemia (B-CLL), indirect (?)
9. AIDS progression, possible role as cofactors
10. Guillain-Barre syndrome
11. Chronic lung disease
12. M-proteinamia
13. Chronic renal failure

Genome of HTLVs. Like any retrovirus, HTLV-I has long terminal repeat sequences at each end. These sets of nucleotides are involved in regulation of the viral gene expression and form sites of covalent attachment to cellular sequences on each side of the integrated provirus.

Like any animal retrovirus, it has the three genes for structural proteins, the *gag* gene for the viral core proteins, the *pol* gene for the enzymes, including RT, and the *env* gene for the envelope (Fig. 3). These give the retrovirus the ability to reproduce itself.

When the molecular analyses of HTLV-I and HTLV-II were completed, it became evident that they have new genes present at the 3′ end of the genome. Originally, one of the genes was called *tat*, but with the revised terminology, this gene is now called *tax*. The *tax* makes a 40000-dalton protein localized in the

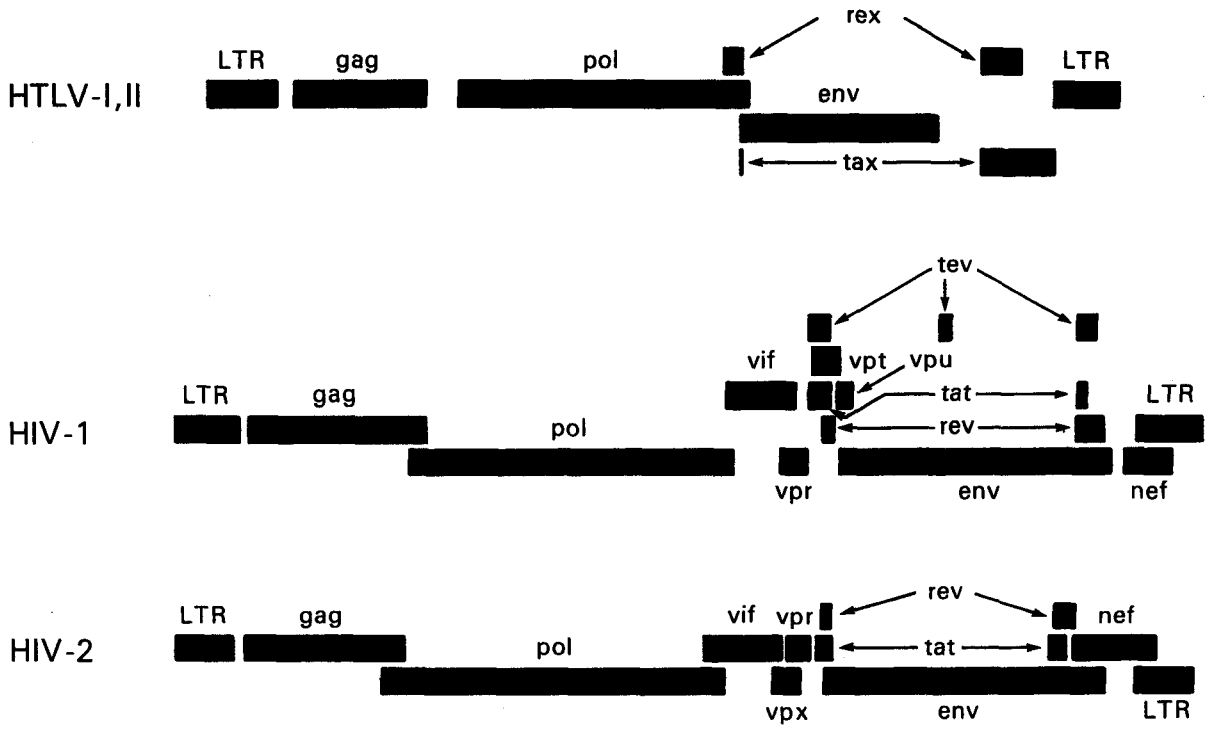

Fig. 3. Genomic structures of human retroviruses

nucleus or the infected cells. The *rex* is the second gene in the 3′ region of HTLV-I and HTLV-II. These genes are coded from two segments of the genome and are products of doubly spliced messenger RNAs. This phenomenon (double or even triple splicing) was new in human retrovirology. It was soon realized that the protein products of these genes are absolutely essential for the replication of HTLV-I and HTLV-II. They are also essential for the biological activity of these viruses.

The products of the *gag*, *pol*, and *env* genes are formed from unspliced or singly spliced messenger RNA molecules. This is similar to what was known among animal retroviruses.

Replication Cycle of HTLVs. The replication cycle of HTLVs can be divided into two parts (Fig. 4). The first part, like any animal retrovirus, involves a phase of attachment to the cell membrane. The receptors for HTLV-I or HTLV-II are unknown, as for most of the animal retroviruses. However, the chromosomal site of the HTLV-I receptor has been determined [21]. Following the attachment, fusion of the viral envelope with the cell membrane occurs, followed by emptying of the viral core into the cytoplasm of the cell. The viral RNA is transcribed in the cytoplasm, with formation of the double-stranded linear DNA, which then enters the nucleus and integrates into the chromosomal DNA.

With most animal retroviruses, after provirus integration into a permissive target cell, virus replication and its expression start immediately. There are sufficient cellular factors, and sufficient viral and cellular machinery allowing quick transcription of the DNA provirus, to reform viral RNA in the nucleus. The viral RNA then traverses to the cytoplasm and assembles at the cell membrane with viral proteins that have been formed by translation of unspliced or singly spliced messenger RNAs in the cytoplasm. The viral proteins are processed, particularly by cleavage through viral and cellular proteases (the former for viral core proteins, the latter for viral envelope proteins). After assembly, budding and release of the newly formed virions completes the replication cycle of the viruses.

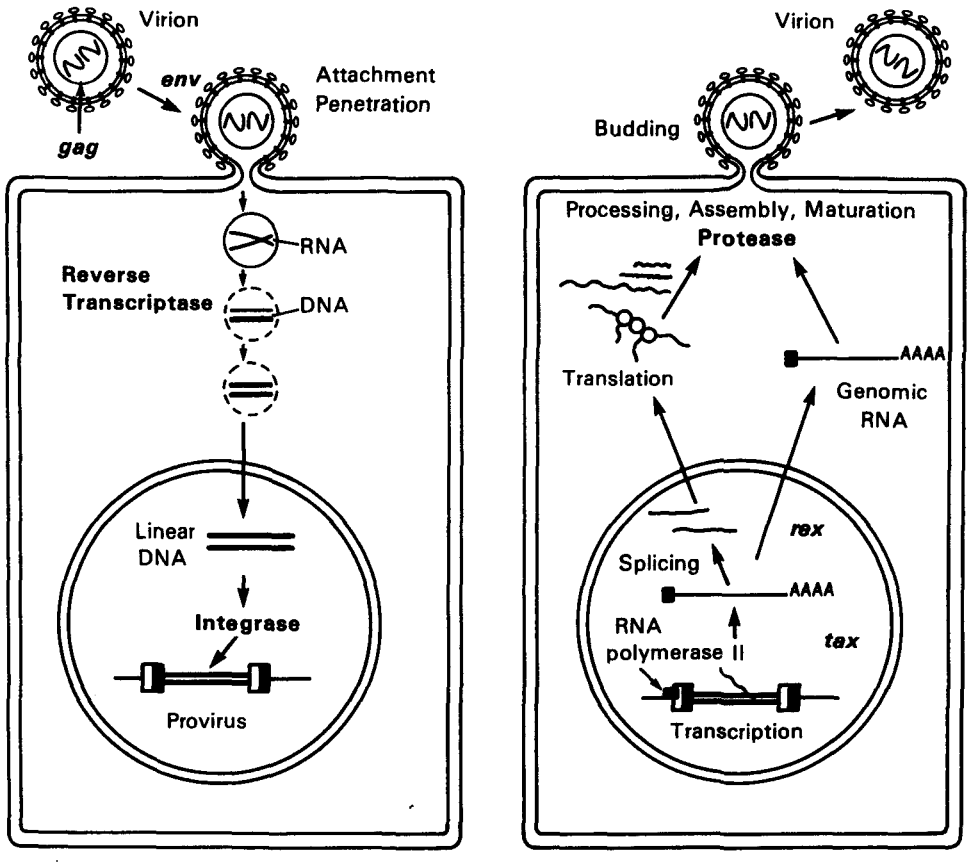

Fig. 4. Life cycle of HTLV-I and HTLV-II

HTLV-I and HTLV-II have introduced a new complexity into our understanding of the replication cycle, and that complexity relates to the events which take place in the nucleus. In order to have successful transcription of the DNA provirus to viral RNA, first there is the expression of an early gene product. This phenomenon, although known in some DNA viruses, was newly discovered in retroviruses. The first genes to be expressed are *tax* and *rex* (Table 2). What turns on the expression of *tax* and *rex* is unknown, but the *tax* gene product (TAX) is essential for the early transcriptional events to make the viral RNA. The function of the *rex* gene product (REX) is not only newly observed in retrovirology, but it has introduced some new mechanisms into all of molecular biology. The REX protein is involved in removal or transport of the messenger RNAs for the viral structural proteins, i.e., the messenger RNAs that are unspliced or singly spliced. In other words, in the absence of REX, the only messenger RNAs that are made are the messenger RNAs that are doubly spliced, i.e., the messenger RNAs for *rex* and *tax*. But once the REX protein is made, the formation of the unspliced RNAs or the singly spliced RNAs for the viral structural proteins is favored. This is an interesting mechanism because once the REX protein is made, it down-regulates its own expression. It also down-regulates *tax* and allows the formation of the viral proteins so that there is a sudden release of virus during this narrow window in which these human retroviruses have to complete their cycle. This mechanism is evident even in HIV but not in the lenti-retroviruses of animals. This may suggest a convergent evolution of mechanisms for infection of human T cells by two entirely different classes of human retroviruses.

Mechanism of Leukemogenesis. TAX protein plays an important role in leukemogenesis. It acts in *trans* and is involved in the mechanism of transcription of viral RNA. TAX protein also activates cellular

Table 2. Accessory genes of human retroviruses

Immunogenicity		Size	Cellular localization	Function	Replication competence of (−) mutants
vif	+	p23	Cytoplasm/inner membrane	Infectivity	±
tat	+	p14	Nucleus/nucleolus	Transcriptional and post-transcriptional activation	−
rev	+	p19	Nucleus/nucleolus	Expression of structural proteins, modulation of transcription	−
nef	+ +	p27	Cytoplasm	Negative regulator	+ +
vpr	+	p18	Nucleus	Rapid viral growth (?)	+ +
vpu (HIV-1)	+	p15	Cytoplasm/membrane	Assembly and release (?)	+ +
vpx (HIV-2)	+	p15	Cytoplasm	?	+ +

genes indirectly. It complexes to some cellular proteins and transcriptional factors that are involved in the turning on of genes important for T-cell proliferation such as those for IL-2 and IL-2 receptor (IL-2R) [22, 23]. It is somewhat ironic that the protein (IL-2) used to grow T cells to isolate the virus is the very protein that the virus uses or turns on in its first stages of leukemia. At least this is the way we think about it today. The TAX protein is also involved in turning on other cellular genes, e.g., the c-*fos* proto-oncogene. The development of adult T-cell leukemia (ATL) by HTLV-I is summarized in Fig. 5. Perhaps about one-third of T cells may be infected by HTLV-I, but only a small fraction expresses the

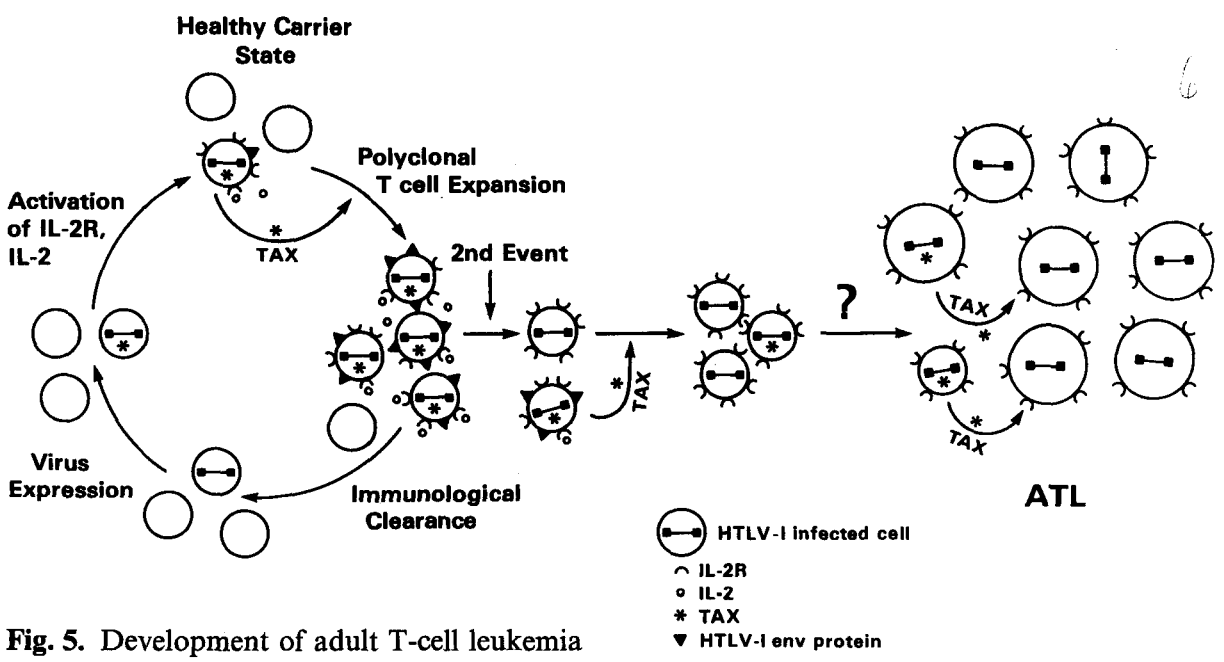

Fig. 5. Development of adult T-cell leukemia

virus. The immune system cannot see the cells which do not express the virus and cannot attack them.

At some stage, the *tax* gene is turned on. What exactly leads to the turning on of *tax* is unknown, but once it occurs genes for other viral proteins can be turned on. The *tax* gene also turns on the IL-2 and IL-2R genes. The IL-2R has a complex structure and is made of different polypeptides. The high-affinity polypeptide of IL-2R that binds best to IL-2 is activated by *tax*.

This may lead to autocrine and paracrine phenomena allowing polyclonal T-cell expansion. At this stage it is not a malignancy but it can be documented in many people infected by HTLV-I. The immune system attacks and clears the proliferating cells expressing viral proteins. The cycles of appearance and clearance of virus-expressing cells occur for years and maybe for decades. The virus continues to increase the expansion of proliferating T cells. As estimated recently in Japan, 3%–5% of the infected individuals will be able to develop monoclonal expansion of a T cell within their lifetime, most likely mediated by another as yet unknown genetic event. This event could be an accident, a mutation, or a rearrangement, but appears to be a chance event ultimately leading to true leukemia. A third genetic event which leads to the blast crisis may be necessary, analogous to chronic myelogenous leukemia. There is no complete agreement on specific chromosomal changes to account for the second or the third event. There are some that are common, but not consistent.

HIV-1 and AIDS

The idea that AIDS might be caused by a $CD4^+$ T-cell lymphotropic retrovirus came from discussions between R. Gallo and M. Essex and his colleagues in Boston who had worked on feline leukemia virus. Discovered in the 1960s by W. Jarrett et al. [24] in Scotland, it was shown by W. Jarrett, O. Jarrett, and others [25] that feline leukemia virus can be transmitted horizontally and cause immune deficiency as well as leukemia. Essex, in his epidemiologic studies in the early 1980s, highlighted the greater importance of this feline virus in immune suppression than in causing leukemia, whereas Gallo suspected from the experiences with HTLVs a possible involvement of a retrovirus in AIDS. These experiences were: studies of HTLV-I epidemiology showed that the AIDS virus was, like HTLV-I, endemic in Central Africa; the causative agent, like the HTLVs, targeted $CD4^+$ T cells; the modes of transmission by sex, blood, and the maternal/fetal route were similar; AIDS was associated with immunosuppression and the HLTVs can be immune suppressive (although modestly); HTLV-II had just been discovered, providing impetus to the idea of there being more human retroviruses. All these things led to thoughts that a new human retrovirus existed perhaps derived from a mutation or a recombinant change in an HTLV-I emerging from Africa, moving to Haiti and then to the United States. This was the notion that led people, ourselves and scientists in Paris, to look for a new retrovirus. However, ironically, we soon learned that, though AIDS is caused by a retrovirus, the virus is not a variant of HTLV-I or a recombinant with HTLV-I, but is due to a different category of human retrovirus(es) that simply has (have) many properties in common, although with a much different genomic organization as well as classification.

There are several components of the overall pathogenesis of AIDS, the major one being the immune deficiency with opportunistic infections. Because of the lifestyle of the individuals there is an increased incidence of infection with real pathogens which include mycobacteria, herpesviruses, HTLVs, and hepatitis and papilloma viruses. In addition, there is infection of the brain in 40%–50% of infected people. Subsequent to infection of the brain, there is a thinking disorder and some acute psychosis. The develop-

ment of two types of tumors is very common (Kaposi's sarcoma and B-cell lymphoma) and must be thought of as involving mechanisms distinct from the other manifestation of AIDS.

Immunodeficiency. The essence of the AIDS problem is immune suppression and immune deficiency. Part of the envelope of HIV, the gp120 molecule, interacts with the CD4 molecule. This interaction has been described as being much tighter and with much greater affinity than many antigen-antibody interactions.

The CD4 molecule is expressed on the surface of cells that are important for the immune system, including T helper lymphocytes, peripheral blood monocytes [26, 27], and cells of the macrophage lineage such as microglial cells of the brain [28], Langerhans cells of skin [29], which are widely distributed in the body, and the follicular dendritic cells of the germinal center of the lymph nodes; this allows the AIDS virus, immediately upon infection, to alter the most pivotal cells of the immune system. An idea to use the soluble CD4 in therapy of people who are infected has already been launched and animal systems are being investigated for that purpose. To use CD4 as a molecular decoy to bind virus before it finds CD4 on the cell surface seems to be the most rational approach to the therapy of this disease. Unexpectedly, however, CD4 is rapidly excreted by humans, and so the results have been extremely disappointing. Much research in the United States and Europe is focused on modification of CD4; for example, Genentech's use of immunoglobulin attached to the CD4 molecule seems to prolong the half-life of CD4, diminishing its rate of excretion.

Genomes of HIVs. The genomes of HIV-1 and HIV-2 are significantly more complex than that of HTLV-I or HTLV-II (Fig. 3). In addition to the three genes that all retroviruses have, *gag*, *pol*, and *env*, HIVs have two regulatory genes called *tat* and *rev* which are analogous to the *tax* and *rex* genes of HTLV-I and HTLV-II and are absolutely essential to the replication of HIVs and probably critical to the biological ability of HIVs to cause AIDS or other manifestations. Many other genes are discovered in the genomes of the AIDS viruses: *vif*, which is essential for cell-free infection by these viruses; *vpr*, which was recently found in our laboratory to be essential for infection of primary human macrophages, but not T cells; *vpu*, whose function is not yet well known; *nef*, which is controversial as to whether it does nothing to virus replication or slightly down-regulates it; and at least two more genes discovered in the last year or so, particularly by Haseltine and his colleagues, whose functions are not yet well understood (Table 2).

The early steps of the life cycle of HIVs are the same as those of animal retroviruses (Fig. 6) and involve attachment and penetration of the virus into the target cell. It is known that the CD4 molecule is the receptor or at least part of the receptor for HIV. Once HIV penetrates the $CD4^+$ T cell, RNA to DNA transcription and DNA integration into the cell follow. Actually, it is not established whether HIV provirus integrates into macrophage. Like HTLV-I and HTLV-II, following integration, there is a silent or latent period even for HIVs. After T-cell activation due to any stimuli, expression of the DNA provirus takes place to form viral RNA and viral proteins. The products of the *tat* and *rev* genes have the same kind of functional corollary as those for *tax* and *rex* of HTLVs. They act as regulatory switches in the replication cycle. In the long silent period after infection nothing happens to the cell if virus is not expressed. But if that cell is immune-stimulated the replication cycle is completed. The virus comes out in a burst, and the cell dies. So, contrary to the notions of some, that retroviruses can not be cytopathic and cytolytic, HIV is certainly cytopathic. Actually, the earlier work of Howard Temin with avian retroviruses also showed the cytopathic and even cytolytic nature of some of those viruses.

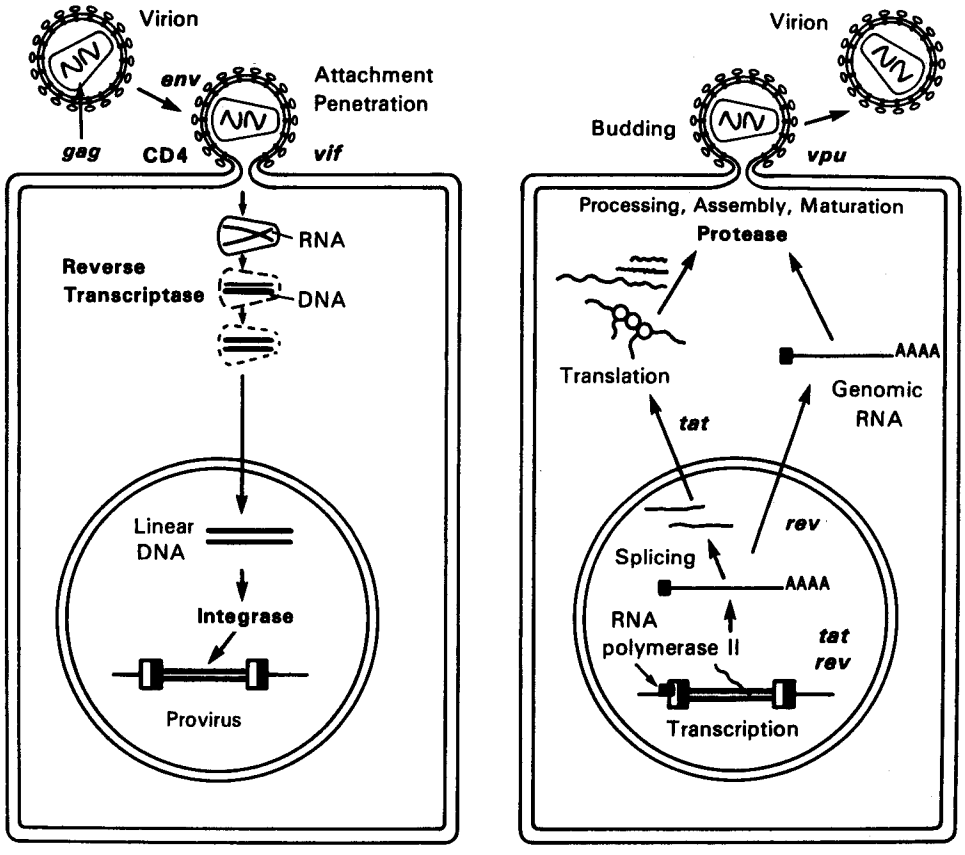

Fig. 6. Life cycle of HIV-1

Mechanisms of HIV Pathogenesis

Role of HIV and HIV Proteins. The question is often asked "Why do the $CD4^+$ T cells become depleted in AIDS?" HIV may be involved in direct killing of infected T cells. HIV also has the capacity to form multinucleated giant cells. When the virus is forming, the envelope protein gp120 is on the cell surface. If there are uninfected cells nearby expressing the CD4 molecule, there will be binding and fusion of the two cells; and this can give rise to fusion of literally several cells together. Such cells have aberrant function and die prematurely. Based on the laboratory observations, one can speculate on other ways which could account for the $CD4^+$ T cells depletion. Extrapolation of these to the in vivo situations may still be remote. It is important to mention that the gp120 falls off the virus easily. In vitro studies show that the gp120 can interfere with T-cell activation. It can also lead to the down-regulation of IL-2 expression in uninfected T cells when this protein binds to CD4 molecules. There are still other indirect mechanisms that could permit the depletion of T cells (see Table 3).

Escape from the Immune System. The infection of the macrophages by HIV shows a very unique feature, namely, infectious whole virus in vesicles inside the cytoplasm of the cell [26]. This happens in only a small fraction of all macrophages.

The important question, however, is: "What if the immune system attacks this cell and destroys it?" Would there be a release of more infectious virus? In laboratory studies the answer is yes. If we take an infected macrophage and we break it manually or by attack from cytotoxic T cells, more infectious virions are indeed released. Therefore, immune therapy that kills infected macrophage must also consider the need for a direct antiviral attack, for example, azidothymidine or neutraliz-

Table 3. Mechanisms of $CD4^+$ T-cell depletion in AIDS

1. Direct killing by HIV following immune stimulation and virus expression
2. Cell death following syncytia formation
3. Decreased IL-2 production
4. Cell-mediated cytotoxicity against uninfected cells mediated by free gp120 complexed to CD4 and antibodies against this complex
5. Some viral protein products inhibit T-cell proliferation
6. Another virus, HHV-6, upon replication is T4 cell-lytic; HHV-6 is common in HIV-infected people and may replicate more in them
7. Defective antigen presentation leads to lower T4 cell proliferation
8. Inappropriate release of certain cytokines, e.g., tumor necrosis factor-α, can decrease T-cell proliferation
9. The gp120-specific class II-restricted cytotoxic lymphocytes can lyse activated ($1a^+$), autologous, uninfected T4 lymphocytes. The CD4 receptor-mediated uptake of gp120 is a critical event for this lytic process. This mechanism could allow destruction of a large number of activated lymphocytes responding to many pathogens

ing antibodies. Neutralizing antibodies against the HIV-1 work by complexing to a certain region of gp120 and blocking entry into the cell.

Virus Variation. Another important question is "Why does the HIV-infected person continue to spread the virus?" We know that HIV varies from person to person. We discovered in 1984 the heterogeneity of HIV for the first time [30]. We found that no two viruses were the same, and the variation was up to 4%–15% in the genomes of different HIV variants. The variation was predominantly in the envelope region. We also showed later that within any one virus isolate there are minor variants. That is to say, if you isolate the virus from one person with AIDS, although most of the viral particles will be very closely related, there is still some variation. And this, in time, has been shown to have biologic significance [31]. For instance, in one virus strain there are many virions with minor differences. At time zero, one variant may predominate and the neutralizing antibody could neutralize almost all of this virus variant. Some time later, another minor variant, perhaps with as little as one amino acid change in the envelop, may emerge, and this may not be neutralized by the original antibody. We have been able to study this in a laboratory worker who, while mass-producing the virus in another laboratory, was infected by accident. One can follow such a person in time. This seems to be an important way by which this virus continues to escape the immune system. Variant-specific antibody develops and can neutralize the virus; new minor variants then emerge, but are not neutralized. One would expect the variation to occur in the region of the neutralizing epitope which exists in the hypervariable region of the envelope. This is true not only with neutralizing antibody, but with cellular immunity as well. In addition to variation in this region, we have seen mutation in completely distant regions, e.g., in the transmembrane region of the envelope protein gp41. Such a mutation also affects the interaction of antibody with this site. More likely the mutations at a distance bring about conformational changes [32, 33].

AIDS and Cofactors. In a small study of homosexuals in Trinidad, Bartholomew et al. studied dual infections with HIV and HTLVs and concluded that HTLV-I may be a cofactor in AIDS [34]. There are additional reports now that agree with this, from Japan in hemophiliacs and from New Jersey with drug addicts [35]. HTLV-I is not needed to get AIDS at all, but the rate of progression may be accelerated in the presence of HTLV-I. Several mechanisms are possible. HTLV-I can lead itself to minor T-cell impairment. The TAX protein of HTLV-I can also activate HIV if the cells are infected

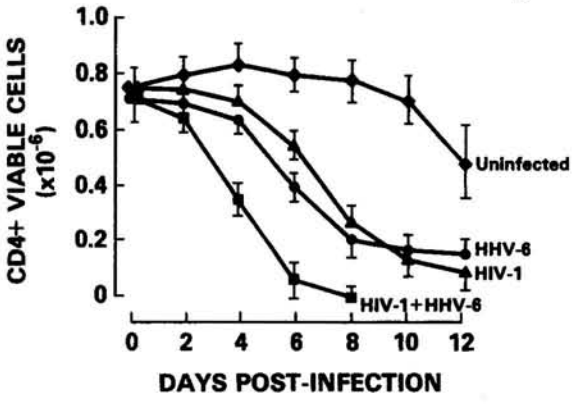

Fig. 7. Killing of $CD4^+$ T cells by HHV-6

with both viruses. In addition, HIV-1-infected T cells can be activated by the simple interactions of HTLV-I with the cell membrane. We have also shown that HTLV-I and HIV can form mixed virus particles, which gives HIV the ability to affect cell types it normally could not affect [36].

The new herpesvirus, human herpesvirus type 6 (HHV-6), which we discovered and isolated in 1986 from B cells [37], actually principally infects $CD4^+$ T cells. This herpesvirus can also kill T cells (Fig. 7) [38]. In the United States, 70%–80% of all people infected with this virus are seropositive. Therefore, in most people, it obviously causes no problem. It is the cause of roseola in babies, which is not a very serious disease. Adults who have antiviral antibodies and cellular immunity can control the replication of the virus. It is possible that in AIDS with immune impairment, there is increased replication of this virus. If so, one must consider damage to the immune system by direct killing of T cells by this virus. In addition, this herpesvirus can activate the HIV genome. It has a gene which makes a protein that can *trans*-activate the expression of HIV [39], analogously to the HTLV-I TAX protein.

Finally, we recently showed that this human herpesvirus is, as far as we know, the only biological agent existing naturally that turns on the CD4 gene at the transcriptional level, and to our knowledge this is the first time it is known that one virus can turn on the receptor of another [40]. In $CD8^+$ T cells and in some epithelial cells, infection by HHV-6 turns on CD4 so that they can become targets for HIV infection. All these aspects of

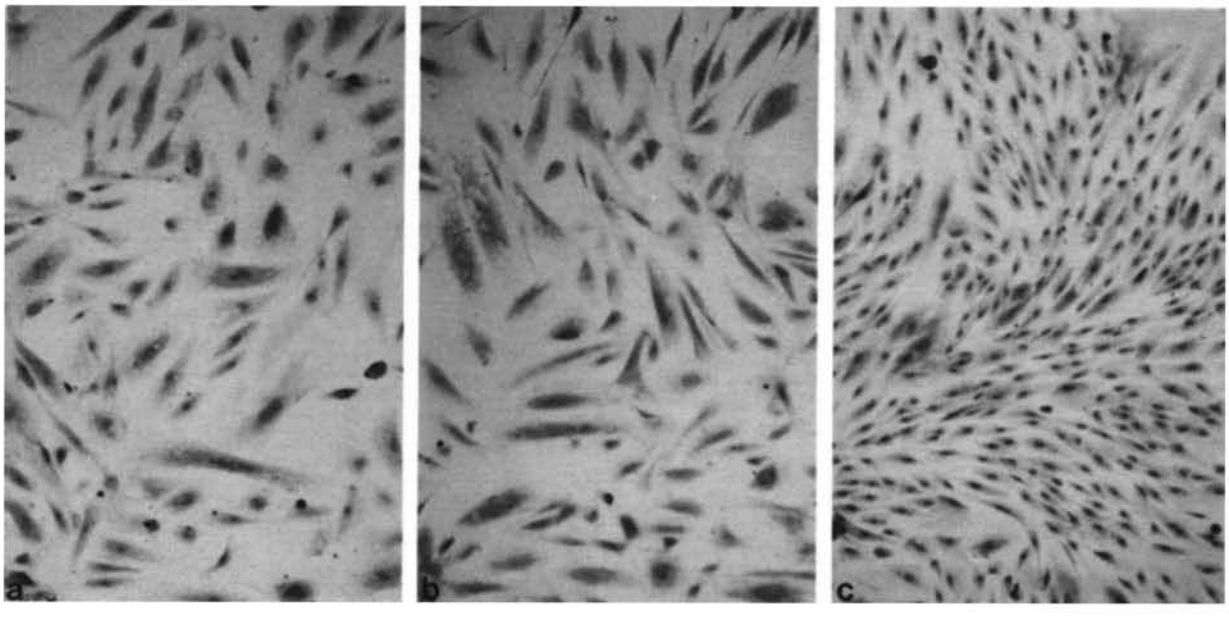

Fig. 8a–c. Spindle cells of Kaposi's sarcoma. **a** Culture in standard medium (RPMI 1640 plus FCS 15%). **b** Standard medium plus endothelial cell growth factor (ECGS) 30 µg/ml and heparin 45 µg/ml. **c** Standard medium plus HTLV-II CM (20% v/v). (From [42], with permission; Copyright 1988 by the American Association for the Advancement of Science)

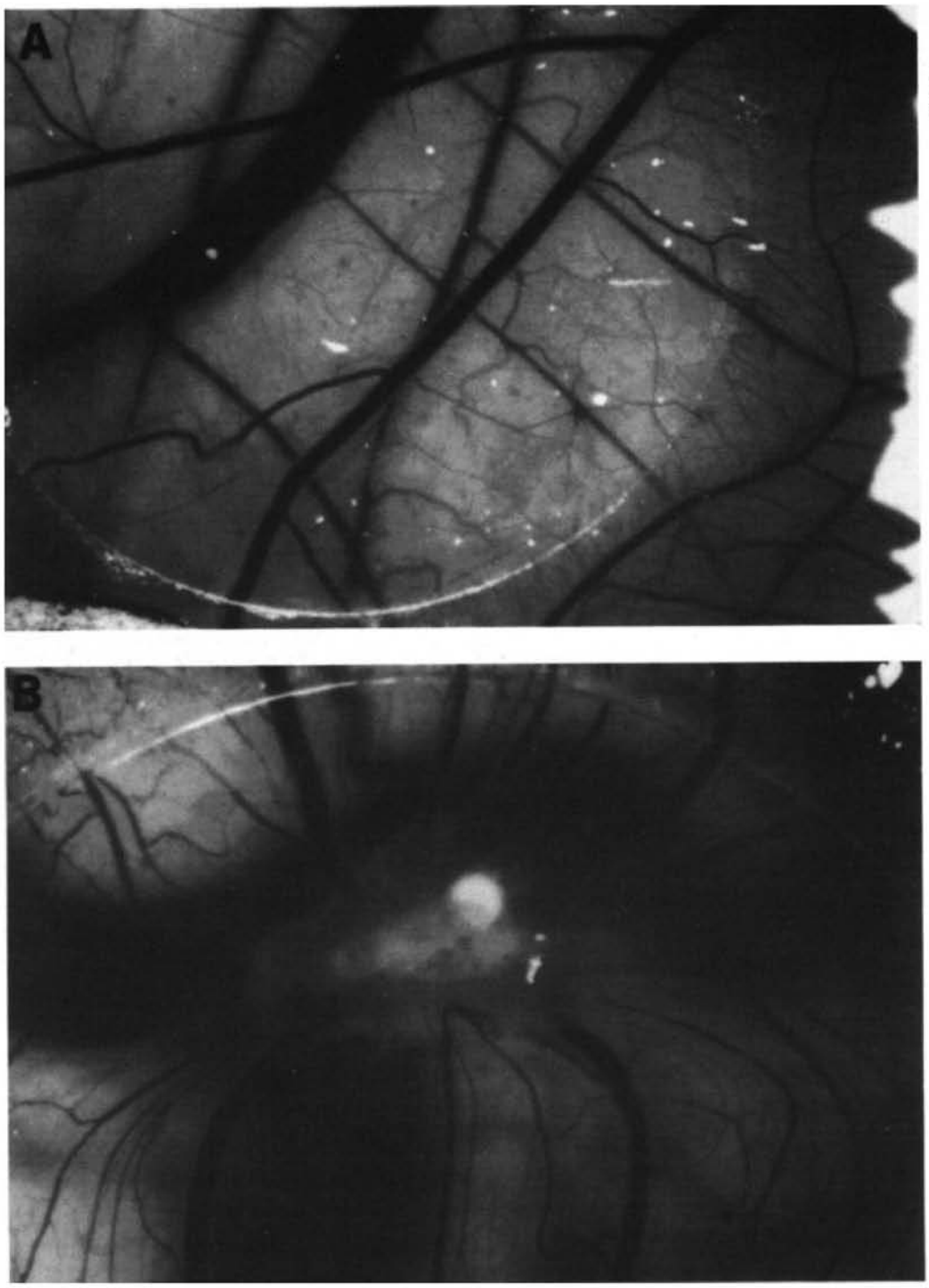

Fig. 9 A, B. Angiogenesis in chick chorioallantoic membrane. **A** Fixed (0.00125% glutaraldehyde) cells gave a negative result. **B** Metabolically active cells gave a strongly positive result. (From [42], with permission; Copyright 1988 by the American Association for the Advancement of Science)

HHV-6 lead us to propose that this virus may contribute to the impairment of the immune system in people already immune suppressed by HIV.

AIDS-Related Kaposi's Sarcoma. Since there has been a great increase in the incidence of Kaposi's sarcoma in HIV-infected people, more so in male homosexuals, one can speculate that HIV infection plays some role. It is not known whether any new or unknown viruses play a role in AIDS-related Kaposi's sarcoma. We started to explore the possibility of other virus(es) but did not find any. In the process, we developed a system for studying Kaposi's sarcoma [41, 42].

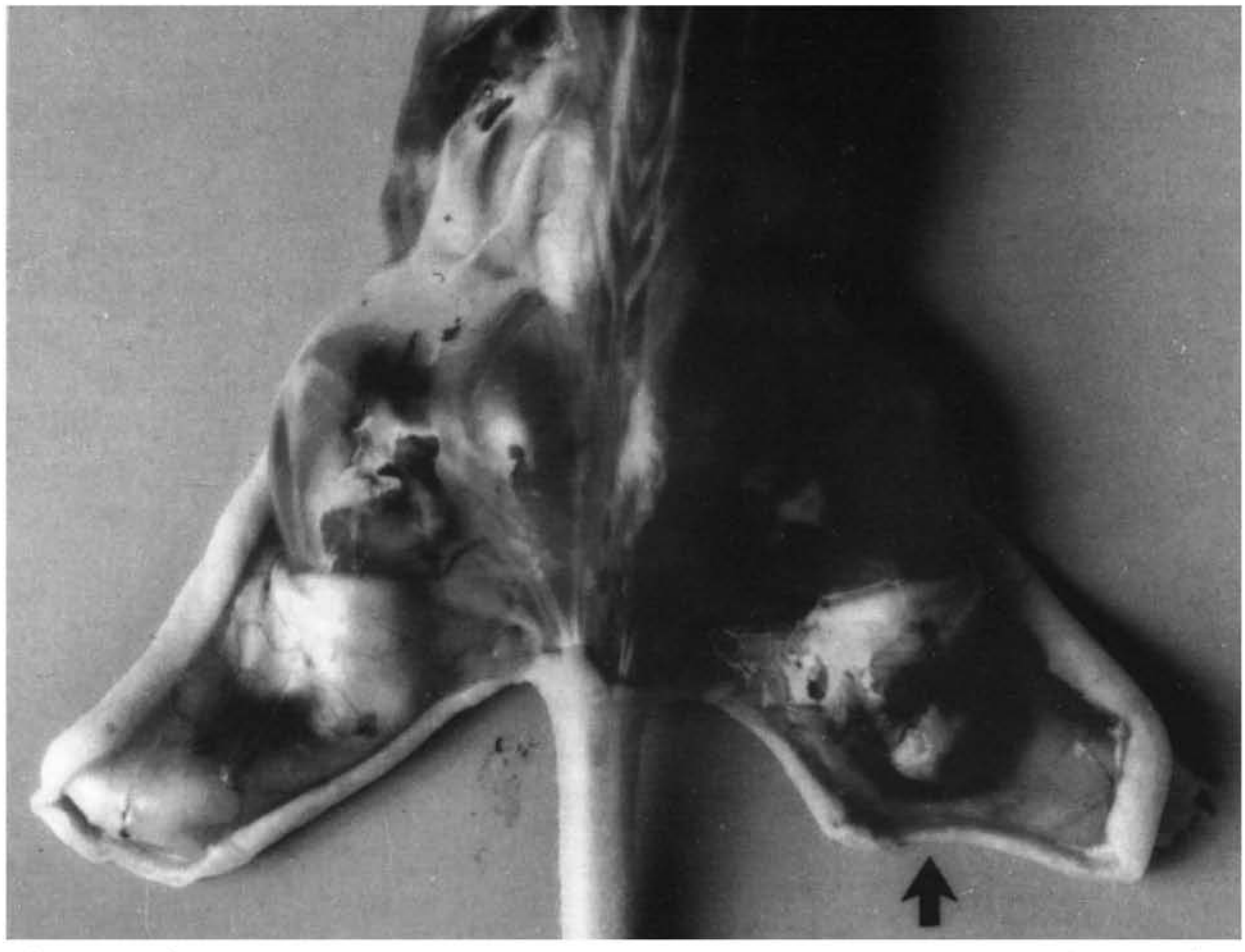

Fig. 10. Lesion in nude mouse induced by Kaposi's sarcoma spindle cells (4×10^6 metabolically active cells injected subcutaneously). *Arrow* indicates positive result. The *left side* was injected with fixed cells

The important thing that came out of our studies over the last few years is that we have a system in the laboratory for studying Kaposi's sarcoma. We can grow the spindle cells which are believed to be the tumor cells of Kaposi's sarcoma. Figure 8 shows the spindle cells derived from a person with Kaposi's sarcoma which were grown for several months in culture. We have several such cell cultures now. These spindle cells have been analyzed in collaboration with Judah Folkman and his associates from Harvard University [43]. They have the properties of primitive smooth muscle cells of vascular origin, as well as some properties of endothelial cells. We think then that the precursor cell of Kaposi's sarcoma is a mesenchymal, primitive precursor of cells of the blood vessel walls. Although we could not find any virus, particularly HIV-1, in these cells, it was found that they release a number of cytokines that have powerful angiogenic activity, which is a key feature of Kaposi's sarcoma. Figure 9 shows angiogenic activity released by the spindle cells grown in the culture tested in the normal chick chorioallantoic membrane [41]. One can take either the intact spindle cells or the concentrate of factors released from them and apply it to the membrane. Distinct angiogenic activity is observed in both instances.

More interestingly, these spindle cells, when put into a nude mouse, cause a tumor similar to human Kaposi's sarcoma to develop (Fig. 10). The lesion develops near the site of inoculation of the spindle cells within 10 days. When the spindle cells regress, the lesion dies out. We examined the lesion histologically. It appears like early Kaposi's sarcoma with blood vessel proliferation, fibroblasts,

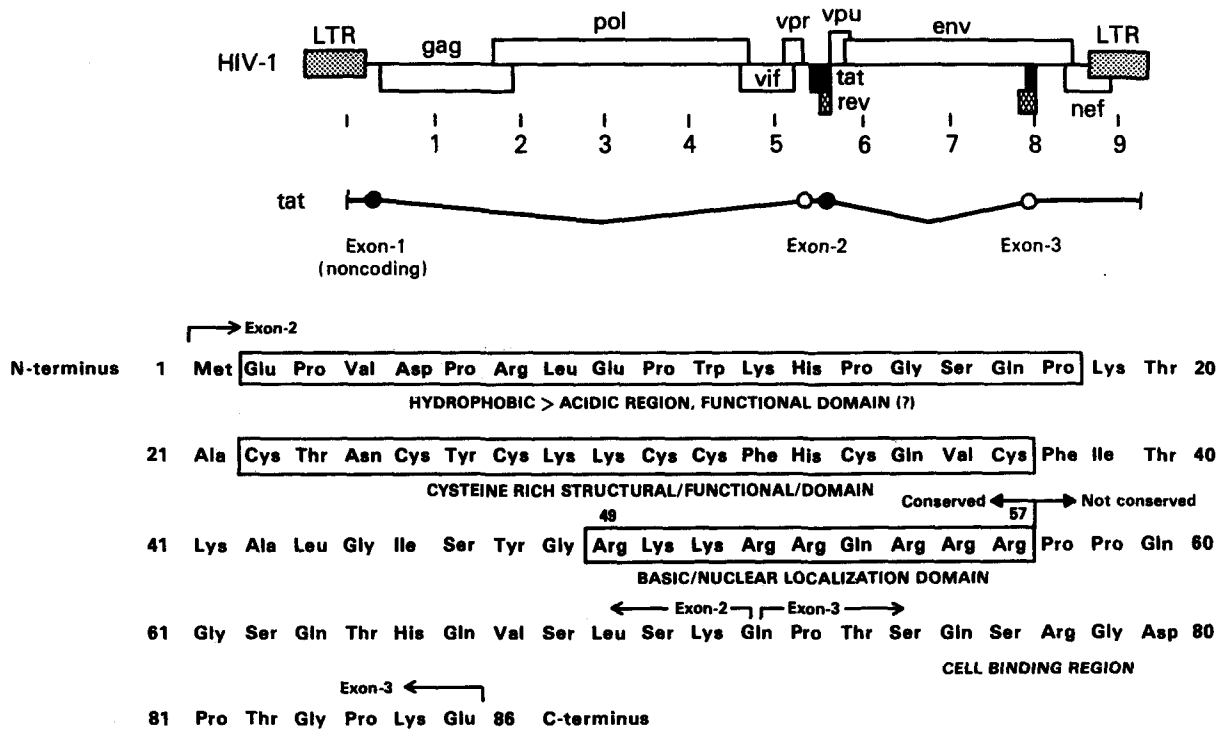

Fig. 11. Different structural and functional domains of the TAT molecule

infiltration with leukocytes, and spindle cells. The conclusion is that the spindle cells secrete factors that are responsible for the early lesion of Kaposi's sarcoma [42]. We have evaluated the cytokines it makes. It appears that IL-1 and basic fibroblast growth factor are the most important ones. These molecules can have angiogenic activity and promote growth of fibroblasts and endothelial cells directly or indirectly. In addition to these, other factors such as granulocyte-macrophage colony-stimulating factor, tumor growth factor-β, IL-6, and low levels of acidic fibroblast growth factor and platelet-derived growth factor are also detected [44].

The manner in which we succeeded in growing the spindle cells is interesting in itself. We grew the spindle cells by using lymphokine(s) made by chronically activated $CD4^+$ T cells. The major active lymphokine for this effect is currently being purified in our laboratory and is the most potent growth factor for AIDS Kaposi's sarcoma spindle cells. In addition, we have found that the TAT protein is released in very small amounts (nanograms) by HIV-1-infected T cells and acts as a growth factor for the spindle cells [45].

The TAT molecule has different regions which are responsible for different activities (Fig. 11). We think one region is particularly important for the growth-promoting activity on the spindle cells [45]. This small protein of 10000 daltons is very complex. It has a region that is important for the *trans*-activation activity of the virus and a basic domain important for the nuclear localization.

Human Retroviruses and Tumorigenesis

The direct effects of a retrovirus like HTLV-I where the virus infects its target cell, can immortalize that cell, and makes it abnormal have been discussed earlier. We find the viral sequences in every cell in the sample place, indicating their clonal derivation from the original transformed cell. HTLV-I can thus be called a directly acting tumor virus. We refer to HIV as having indirect effects that can lead to the increased possibility of tumor develop-

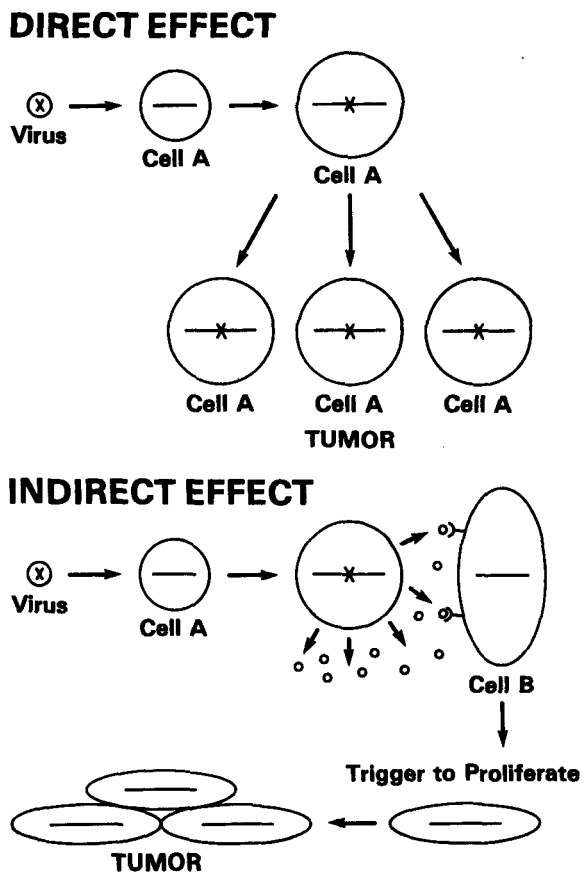

Fig. 12. Direct and indirect mechanisms of tumor induction by human retroviruses

ment. HIV probably increases the possibility of Kaposi's sarcoma developing in at least two ways:

1) it infects T cells and releases TAT protein;
2) its proteins activate immune cells (T cells and B cells) which release lymphokines.

Some of these lymphokines can have an effect on the primitive mesenchymal cell that has lineage to smooth muscle and endothelium and which is the precursor of the spindle cell of Kaposi's sarcoma. This cell in turn releases a series of cytokines that act to form a complex mixed tumor that we call Kaposi's sarcoma. In summary, human retroviruses can induce tumors, directly or indirectly, in addition to their suppressive effects on the immune system and abnormal effects on the nervous system (Fig. 12).

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