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THE BIOCHEMISTRY OF AIDS

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CONTENTS

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I. HISTORICAL PERSPECTIVES ON HUMAN RETROVIRUSES

Retroviruses are RNA-containing viruses that replicate through a DNA intermediate by virtue of a viral-coded RNA-dependent DNA polymerase, also called reverse transcriptase (1, 2). The family of retroviridae is divided into three subfamilies (3). Oncovirinae includes all the oncogenic retroviruses and many closely related non-oncogenic viruses. Lentivirinae includes the "slow" viruses such as visna-maedi virus, Caprine Arthritis Encephalitis Virus (CAEV), Equine Infectious Anemia Virus (EIAV), and the immunodeficiency viruses, including most notably Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immunodeficiency Syndrome (AIDS) (4–6). The third subfamily, spumavirinae, consists of the "foamy" viruses that induce persistent infections without any clinical disease but cause vacuolization of cultured cells (3).

Recognition of a viral etiology of cancer dates back to the early 20th century when the transmission of chicken leukemia or sarcoma was accomplished through injection of filtered tumor extracts (7–9). The first mammalian retrovirus, murine leukemia virus, was isolated by Ludwik Gross in 1951 from an inbred mouse strain (10), which provided the first reproducible experimental system to study viral leukemogenesis. In the ensuing period, retroviruses were shown to cause lymphomas, leukemias, sarcomas, and various other cancers in species ranging from chicken, mice, and cattle to subhuman primates (2). The discovery of reverse transcriptase in 1970 by Temin & Mizutani (11), and independently, by Baltimore (12) substantiated the "provirus hypothesis" proposed by Temin in 1964 (13) and also established a genetic and biochemical basis for the classification of retroviruses.

With the role of retroviruses in carcinogenesis firmly established in animal models, massive efforts were then directed towards the search for human retroviruses in similar diseases (for reviews, see Refs. 14 and 15). Various approaches were taken towards the detection of retroviruses in normal or malignant human tissues: electron microscopy, detection of reverse transcriptase activity, study of virus-like RNA-protein complexes, evaluation of antigen or nucleic acid sequence relatedness to known animal retroviruses, rescue of defective viruses, and observation of reactivity of patients' sera with retroviral proteins. None of the above studies yielded unambiguous results. Parallel studies in the bovine leukemia virus model (16) provided valuable reminders that retroviral-induced diseases need not be associated with overt viremia, nor is the etiological agent necessarily related to previously known animal retroviruses and therefore detectable by probes derived from them. Appreciation of these possibilities, and of the importance of long-term culture of tumor cells in vitro, were critical for the isolation of human retroviruses.

The isolation of the first human retrovirus, human T-cell leukemia virus I (HTLV-I) (17) came on the heel of the discovery of interleukin 2 (IL-2), which enabled long-term cultivation of mature T-cells of normal and neoplastic origins (18). Both accomplishments took place in the laboratory of Gallo and associates. Although HTLV-I was first isolated from a patient diagnosed with mycosis fungoides, the virus had no etiological association with the disease. The establishment of a link between HTLV-I and malignancy rested on the recognition of a new disease entity termed "Adult T-cell Leukemia/Lymphoma" (ATLL), which clusters in parts of southern Japan (19, 20) and was suspected to have a viral etiology. ATLL sera were found to be almost 100% positive for reactivity against HTLV-I (21, 22). A similar virus was subsequently isolated from ATLL patients and was designated as adult T-cell leukemia virus (ATLV) (23). Since HTLV-I and ATLV were practically identical viruses (24), the name HTLV-I was adopted as a consensus designation. The second human retrovirus, human T-cell leukemia virus type-II (HTLV-II), also was isolated by Gallo's team in 1982 from a patient with a T-cell variant of hairy cell leukemia (25).

In spite of HTLV-I and HTLV-II, human retroviruses would probably have remained relatively obscure in the United States and Europe had it not been for the sudden emergence of another retroviral disease that reached epidemic proportions. In 1981, five cases of *Pneumocystis carinii* pneumonia (PCP) occurring in previously healthy homosexual men were described in the United States (26). Outbreaks of this and other immunodeficiency-associated conditions such as Kaposi's Sarcoma, mucosal candidiasis, etc, soon followed (27–30). Strikingly, all the patients were either homosexual men or intravenous drug abusers and all had a decreased T-cell response to mitogens and antigens. The term Acquired Immunodeficiency Syndrome (AIDS) was coined to define clinically the various manifestations of this disease. By the end of 1982, the number of AIDS cases throughout the United States had exploded, and the disease was no longer restricted to homosexuals and drug abusers but also extended to hemophiliacs, blood transfusion recipients, Haitian immigrants, sex partners of risk-group members, and children born to mothers at-risk.

All the observations strongly suggested the etiological involvement of a transmissible agent spread through genital secretions and blood. A new retrovirus was an attractive idea since some animal retroviruses, e.g. feline leukemia virus (31), were known to induce immunodeficiency as well as leukemia/lymphoma. There were also some similarities between the previously discovered human retroviruses (HTLV-I and HTLV-II) and the putative AIDS agent with respect to host cell tropism and modes of transmission. In 1983, Barre-Sinoussi et al detected reverse transcriptase, cytopathic activity, and virus particles in phytohemaglutinin (PHA)- and IL-2-stimulated

lymphocytes from a patient with lymphadenopathy, a frequent prodrome of AIDS (32). The virus was designated as LAV (lymphadenopathy-associated virus). Other similar cultures were subsequently reported (33). The causal association between LAV and AIDS remained equivocal, however, because no purified virus reagent was available to type the virus from the various cultures or the sera of different AIDS patients. A team headed by Gallo reported in 1984 the first long-term propagation of viruses from several AIDS patients in permanent $CD4^+$ T-cell lines (34, 35). The availability of continuous, high-titer producer cell lines allowed for the first time the development of highly purified and concentrated viral reagents necessary for the characterization of the virus and for the serological detection of exposed individuals (36, 37). Because of the tropism of this virus, Gallo et al designated it HTLV-III. Another team headed by Levy reported the isolation of similar viruses, which were designated as AIDS-related viruses (ARV) (38). Later on, HTLV-III, LAV, and ARV were found to be variants of the same AIDS virus (39). In 1986, an international committee recommended the name human immunodeficiency virus (HIV) for all isolates of the AIDS virus (40).

Since AIDS has become a global medical emergency, the scientific community has responded to the challenge by making progress at an unprecedented rate in the characterization of the viral agent. As a result, the life cycle of HIV is now understood more fully than that of any other retrovirus. Antiviral agents have been developed with some success, some of which may be applicable to other retroviral diseases of animals and humans. Massive efforts to develop an HIV vaccine are beginning to pay off. Furthermore, an unforeseen dividend from HIV research is the unraveling of the rich, intricate pathways of gene regulation utilized by the virus, which may well illuminate novel, fundamental cellular processes. The understanding of basic biology that we gain from the studies of HIV may be one major legacy of this epidemic to medical science.

II. BIOLOGICAL PROPERTIES OF HIV

A. Target Cell Tropism

One of the conspicuous features of HIV infection is a selective depletion of CD4-bearing (helper/inducer) T-lymphocytes, suggesting a selective tropism and cytopathic effect of HIV for this population. This suggestion was supported by in vitro demonstration that purified $CD4^+$ T-cell lines preferentially support the replication of HIV (41) and that syncytia formation and cell death are frequently associated with infection. Furthermore, it was shown that both infection and syncytia formation could be blocked by monoclonal antibodies directed against certain epitopes of the CD4 molecule (42–44). The HIV-1 envelope glycoprotein gp120 binds to CD4 as indicated by their coprecipita-

tion by antibodies directed against either of the proteins (45). The definitive proof that CD4 is a receptor for HIV was provided by Maddon et al (46) when they showed that $CD4^-$ cells (such as HeLa), which are ordinarily not targets for HIV infection, could be rendered infectable by transfection of a cloned CD4 gene (46). Interestingly, expression of human CD4 is not sufficient to confer infectivity to murine cells, suggesting that a second component present in human cells but not in murine cells is required for postbinding events in the infection process. Finally, soluble CD4 inhibits binding and infection of both HIV-1 and HIV-2 in vitro (47–49). In addition to helper T-cells and monocyte-macrophages (50–52), other cells such as Langerhans cells (53), follicular dendritic cells (54), glial cells (55, 56), and certain colon tumor cell lines (57, 58) are susceptible to HIV-1 infection. These cells express low levels of CD4 on their surface. Similarly, five hepatoma cell lines have been shown to be infected productively by HIV-1 despite the absence of detectable CD4 expression (59). Moreover, soluble CD4 and monoclonal antibodies against CD4 failed to block HIV-1 infection in these cell lines. However, the nature of an alternative receptor suggested by these studies remains to be determined.

B. Syncytia Induction and Cytopathic Effect

Various mechanisms have been proposed to explain the cytopathic effects (CPE) of HIV on T-cells, particularly to accommodate the fact that a dramatic depletion of T-cells occurs even though relatively few cells are actually infected (60). One possible mechanism is through syncytia formation, which involves interaction of HIV-infected cells expressing viral glycoproteins (gp120 and gp40) on the cell surface and uninfected cells expressing CD4 (61–63). The syncytia are unstable in culture, do not proliferate, and usually die within 48 hours. In this way, a few infected cells may form the nuclei of many dying cells. However, syncytia formation is unlikely to be the major pathway for cell loss, as some cell lines that do not form syncytia are nevertheless susceptible to CPE and conversely, syncytia formation could be transient and not lead to significant cell death. Intracellular complexing of CD4 and gp120 or destruction of the permeability of the membrane owing to profuse virus budding have also been proposed to play a role in the single cell killing by HIV (64).

Like other cytopathic lentiviruses, HIV accumulates large amounts of unintegrated viral DNA upon infection (65). Although this feature has been suggested as an important factor in HIV-induced CPE (65), comparison with a mutant virus with greatly reduced cytopathicity did not reveal apparent differences in the level of unintegrated DNA (66).

Additional mechanisms of CD4 cell depletion can be invoked. For example, extracellular gp120 (shed by infected cells) may bind to CD4 ex-

pressed on the surface of uninfected T-helper cells and make the cell a target for antibody-dependent cell-mediated cytotoxicity (ADCC) (67). Other conceivable mechanisms include autoimmune phenomena directed against infected lymphocytes and HIV-induced terminal differentiation of infected cells.

Monocytes and macrophages have been shown to harbor HIV-1 in vivo and are likely to play an important role in the pathogenesis of the virus (50–52, 68). Monocytes/macrophages are relatively resistant to HIV CPE, probably due to the low level of CD4 expression. Therefore, monocytes may act as a reservoir of virus and may disseminate virus to various organs in the body. A noncytopathic, low-level infection of monocytes is also seen with other lentiviruses such as the visna virus of sheep (69). Also analogous to visna virus infection, monocytes appear to be the predominant cell type infected by HIV within the central nervous system of infected individuals (70–72). Infection of this target cell could be the basis of neurological abnormalities that are seen, to varying degrees, in at least 60% of AIDS patients. Lee et al (73) found that gp120 could inhibit the growth of neurons in the presence of neuroleukin, but not in the presence of nerve growth factor. It was postulated, based on the observed partial sequence homology between gp120 and neuroleukin, that gp120 could act as a competitive inhibitor of neuroleukin. AIDS-associated dementia could probably be explained by the observation that free gp120 even at low concentration could increase intracellular free calcium in rodent retinal ganglion cells and hippocampal neurons in culture and cause cell death within 24 hours (74). The specificity was evident as the effect could be abolished by antibodies against gp120. The mechanism of action is not clearly understood. The action of gp120 may be through a receptor, via a second messenger, or directly on Ca^{2+} channels.

C. Clinical Manifestations of HIV Infection

$CD4^+$ helper T-cells not only are quantitatively depleted but also are functionally abnormal in AIDS patients (75, 76). Other cells of the immune system are also affected, directly or indirectly, leading to the development of immunodeficiency which in turn allows opportunistic infections of a variety of agents. *Pneumocystis carinii* pneumonia (PCP) is the most common opportunistic infection in AIDS (77), and it appears to represent activation of latent infection in the majority of cases (78). Other opportunistic pathogens include *Candida albicans, Mycobacterium avium* complex, *Cryptococcus neoformans, Histoplasma capsulatum,* and papilloma viruses, all of which may cause skin lesions in HIV-infected persons. Reactivation of many other DNA viruses, including the herpesviruses, papovaviruses, and hepatitis virus, is also frequently seen. Another pathological consequence of HIV infection is the high incidence of Kaposi's Sarcoma (KS), particularly in homosexual

males. A relatively benign form of KS has been previously observed in older Mediterranean men. KS in AIDS patients may involve skin and mucous membranes. Neurological manifestations could be a direct consequence of HIV infection in the brain, or due to opportunistic infections such as toxoplasmosis, cryptococcosis, JC papovavirus infection, and mycobacterial infections. For a more extensive account on the clinical aspects of HIV infection, please consult the review by Hirsch & Curran (79).

III. REPLICATION CYCLE OF HIV

A. Virus Binding, Fusion, and Entry

HIV is related to the animal lentiviruses on the basis of genetic, morphological, and pathological criteria. Electron microscopy has revealed a characteristic cylindrical core in HIV particles which is indistinguishable from that of animal lentiviruses (4) (Figure 1). The diameter of HIV virions is approximately 110 nm. Like other retroviruses, HIV has two copies of 35S single-stranded genomic RNA with the polarity of mRNA. The genomic RNA supposedly exists in the form of a ribonucleoprotein complex containing reverse transcriptase (p55/p61), endonuclease (p32), and retroviral RNA-binding Gag protein (p9). The viral core is composed of p24 as a major capsid antigen. The myristylated Gag protein (p17) forms the outer shell, and its amino-terminal end is inserted into the lipid membrane envelope derived from the infected cell membrane. The extracellular (gp120) and transmembrane (gp41) glycoproteins exist in the form of a noncovalent complex on the envelope.

The first step in the initiation of infection is the binding of a virus particle to a specific receptor on the surface of the target cell. In the case of retroviruses and other enveloped viruses, the interaction with the cell receptor is mediated by the envelope glycoprotein. As discussed earlier (Section IIA), the receptor for HIV is CD4, and the gp120 of HIV binds to CD4 on the target cell with an affinity constant of the order of 10^{-9} M (80). CD4 is not only the first retroviral receptor to be identified, but is also the most extensively studied. The only other retroviral receptor gene that has been identified, cloned, and sequenced is that of Molony murine leukemia virus (81). Mutational studies have implicated the amino-terminal half of gp120 in mediating the association with gp41 transmembrane protein in a head-to-head configuration and the carboxyl-terminal region in interaction with the CD4 receptor (82). The epitopes on gp120 recognized by murine monoclonal antibodies capable of inhibiting the interaction between gp120 and CD4 have been mapped to a relatively conserved region near the carboxy terminus spanning amino acids 397–439 (83). The deletion of 12 amino acids as well as a single amino acid substitution in this region resulted in a complete or substantial loss of binding

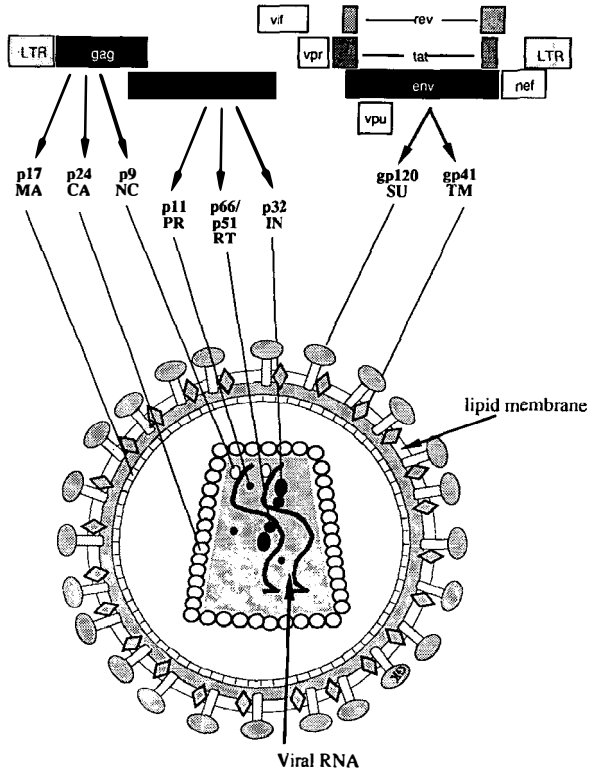

Figure 1 Structure of human immunodeficiency virus (HIV).

to CD4. Similarly, the binding site for gp120 has been mapped to a portion of the first amino-terminal variable domain of CD4 receptor (amino acid residues 16–84) that encompasses the region homologous to the second complementarity-determing region (CDR2) of immunoglobulin variable region (84–87). Additionally, amino acid residues of the second variable domain may contribute to gp120-binding (84, 86).

The envelope glycoprotein of most enveloped viruses exists as a heterodimer, often held by interchain disulfide bonds, with the extracellular component mediating the interaction with receptor and the transmembrane component inducing fusion with the host cell membrane. By analogy, the transmembrane protein gp41 of HIV has been thought to mediate fusion between the viral envelope and host cell membranes (82, 88). The transmembrane envelope glycoprotein of orthomyxoviruses and paramyxoviruses generally contains a stretch of hydrophobic amino acids at the amino terminus, which has been implicated in viral membrane fusion (89). HIV-1 gp41 also contains such a

hydrophobic amino-terminal sequence. Linker insertion as well as point mutations in this region were found to abrogate cell fusion (82, 90). Single polar amino acid substitutions introduced throughout the stretch of 28 hydrophobic amino acids of gp41 dramatically reduced syncytia formation, suggesting a role for the entire hydrophobic terminus in cell fusion (91). Amino acid substitutions have also been introduced in the 15 amino-terminal residues of a simian immunodeficiency virus (SIVmac) gp32 transmembrane glycoprotein. It was found that mutations that increased the overall hydrophobicity of the gp32 amino terminus increased the ability of the viral envelope to induce syncytia formation, whereas introduction of polar or charged amino acids in the same region abolished the fusogenic function of the viral envelope (92).

The mode of entry of enveloped viruses can be broadly divided into pH-dependent and pH-independent processes (89). The former mode involves internalization of virus by receptor-mediated endocytosis into acidic compartments (endosomes), where low pH induces conformational changes in the transmembrane envelope glycoprotein, as a result of which the hydrophobic fusion domain is exposed, which in turn facilitates fusion of viral and endosomal membranes. As an example, this mechanism is used by influenza virus, an orthomyxovirus. The second mode, which is used by Sendai virus, a paramyxovirus, involves direct fusion of the virus envelope with the plasma membrane of the cell in a pH-independent manner. To distinguish these two modes, lysosomotropic agents such as weak bases (ammonium chloride, chloroquine, and amantadine) and carboxylic ionophores (monensin and nigericin), have been instrumental by virtue of their ability to neutralize the acidity of endosomes and thereby inhibit only the pH-dependent entry. In the case of HIV, the events that follow binding are less clear (for a review see Ref. 93). HIV infection was first reported to be inhibited by ammonium chloride, implying the pH-dependent mode of entry (46). However, it was later reported that ammonium chloride, chloroquine, amantadine, and monensin do not inhibit entry of HIV, suggesting that HIV does not require low pH for fusion (94, 95). Furthermore, electron microscopy has revealed direct fusion of the virus envelope with the plasma membrane (94). The fact that HIV-1-induced syncytia formation can occur in media maintained at neutral or mildly alkaline pH also supports the view that HIV enters susceptible cells via pH-independent membrane fusion. Mutant CD4 receptor with deletion of cytoplasmic domain was found to function as an effective receptor for HIV even though it was not internalized in response to phorbol esters (96). These results suggest that receptor-mediated endocytosis is not required for HIV entry. However, the possibility that the truncated CD4 molecules are still internalized in response to HIV binding cannot be excluded. Alternatively, internalization may still proceed independent of the cytoplasmic domain through an association between CD4 and another plasma membrane protein.

B. Synthesis and Integration of Proviral DNA

After entry of a retroviral core into the cytoplasm of a susceptible cell, viral RNA is transcribed into double-stranded proviral DNA by the RNA/DNA-dependent DNA polymerase and ribonuclease H activities of reverse transcriptase. The proviral DNA, presumably in the form of a nucleoprotein complex, then migrates to the nucleus where it is integrated into the host cellular DNA by the endonuclease activity of the viral integrase, with possible participation of cellular proteins (for a review see Ref. 97). By analogy with animal retroviruses, HIV proviral DNA synthesis and integration are presumed to follow the same pathway. Retroviruses in general do not replicate in nondividing cells (98). Similarly, HIV has also been shown to require activated T-cells for productive infection in vitro (99, 100). HIV-1 infection of quiescent primary lymphocytes is stable but nonproductive until after mitogenic or antigenic stimulation (100–102). The restriction, the mechanism of which is unclear, appears to be at the level of complete proviral DNA synthesis. It has been shown that HIV-1 enters and initiates DNA synthesis in quiescent T-cells at levels comparable with those of activated T-cells. However, unlike that of activated T-cells, the viral RNA is not completely reverse transcribed in quiescent cells (103). The incompletely transcribed proviral DNA contains both minus and plus strands of one long terminal repeat (LTR) (3') with some further limited extension of the minus strand. This structure is labile with a half-life of about one day. The incomplete proviral DNA may account for the failure of some investigators to detect HIV-1 DNA in quiescent T-cells by Southern blot analysis, which was taken as an evidence that T-cell activation is required for virus entry (104). Another report suggested a defect at the level of proviral integration in the resting T-cells, which were found to contain a transcriptionally active extrachromosomal form of HIV-1 proviral DNA (105). The latter could persist for several weeks in resting T-cells and, upon T-cell activation, could integrate and act as a template for infectious virus. However, the requirement of cell proliferation for productive infection is not universal. For example, nondividing cultures of monocytes and macrophages can be infected by HIV. Furthermore, the PBJ strain of SIV, which is highly virulent in macaque monkeys, is capable of establishing productive infection in resting T-cells, while closely related SIV strains are not (P. Fultz, personal communication). A second level of restriction for virus replication is at the level of initiation of transcription, as some of the factors required for HIV promoter activity, such as NFκB, are activated upon mitogenic/antigenic stimulation. A detailed discussion of various factors interacting with HIV LTR is given in Section IIIC.

Most aspects of integration of HIV appear to be similar to other retroviral systems. For example, HIV-integrated DNA contains highly conserved dinucleotide sequences (5' TG—CA 3'), and there is a duplication of a short

stretch of cellular DNA at the site of integration. Moreover, HIV viral termini contain short inverted repeats, which may be important for specificity of integrative recombination with respect to the viral DNA. Integration of HIV-1 DNA in vitro into heterologous DNA targets using extracts of cells infected with HIV-1 has been demonstrated (106, 107). As in the case of other retroviruses (97), the viral DNA active in integration was found to exist in the form of a nucleoprotein complex. Moreover, integration of linear HIV DNA occurred in cytoplasmic extracts that contained no detectable circular forms of viral DNA (106), consistent with other retroviral systems, in which linear DNA has been shown to be the immediate precursor for integration (97).

Whether proviral integration is necessary for HIV replication is yet to be established. Cells infected with HIV-1 carrying deletion in integrase gene were found to produce core and envelope antigens, but not infectious virus particles (105).

C. Expression of Viral Genes

Once the provirus is integrated into the host DNA, it behaves as a resident cellular gene. For most retroviruses, the expression of integrated provirus follows the same general rules of eukaryotic gene expression and is governed exclusively by cellular proteins (97). The gene expression of human retroviruses (HIVs and HTLVs), however, is additionally controlled by viral regulatory proteins. Transcription is a complex process involving interplay between *cis*-acting regulatory sequences present in the viral LTR and *trans*-acting cellular transcription factors as well as viral transactivators (e.g. Tat of HIV and Tax of HTLV). The regulatory sequences present upstream of the transcription initiation site (+1) can be broadly catagorized into proximal (promoter) and distal (enhancer) elements (Figure 2). They are sequences typically utilized by cellular RNA polymerase II. The promoter element contains the TATA box, located between nucleotides −22 to −27, which is presumably recognized by transcription factor TFIID (108). The TATA sequence, the most highly conserved sequence in the RNA polymerase II–transcribed genes, determines the precise site of transcription initiation. The HIV-1 promoter also contains three GC-rich sequences, which bind to the ubiquitous transcription factor Sp1 (108, 109). Binding of Sp1 increases the rate of transcription initiation.

The enhancer is a distal region that stimulates transcription initiation in a distance- and orientation-independent manner. HIV-1 LTR contains such an element between nucleotides −120 and −57 (110). The enhancer also appears to govern the induction of HIV promoter activity in response to T-cell activation. It is recognized by EBP-1 in resting cells (111) and by NFκB (112) and HIVEN 86A (113) in activated T-cells. There are two binding sites (−109, −79) for NFκB in the form of imperfect repeats. Binding of purified

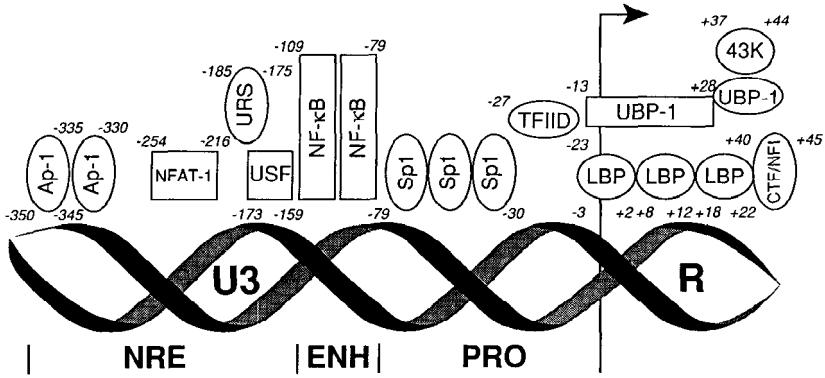

Figure 2 Organization of HIV-1 long terminal repeat (LTR). Binding sites of various factors are shown along with the coordinates of the recognition sequences. See text for details. NRE, negative regulatory element; ENH, enhancer; PRO, promoter. The approximate boundaries of NRE, ENH, and PRO are depicted by vertical lines. The arrow indicates the site of transcription initiation.

NFκB to HIV 1-LTR has been demonstrated in vitro (114). In resting T-cells, NFκB is present in the cytoplasm complexed with an inhibitory protein, IκB. Upon stimulation, NFκB is released from IκB and consequently translocated to the nucleus (for review see Ref. 115). The induction of HIV promoter activity in response to T-cell activation has been primarily attributed to the binding of NFκB (112, 114, 116). Activation of T-cells also induces a T-cell-specific protein HIVEN 86A (113). However, it is not clear whether the binding of NFκB and HIVEN 86A is simultaneous or mutually exclusive.

Recently, intragenic enhancers have also been reported in several retroviruses. A sequence with enhancer-like activity has been found in the *gag* region of avian leukemia-sarcoma viruses (117–119). Similarly, HIV-1 also contains an intragenic enhancer in the *gag-pol* region (between nucleotides 1711 and 6026) (120). However, the role of these intragenic enhancers in the initiation of transcription from the LTR is speculative at present.

The sequence located between nucleotides −410 and −157, designated as the negative regulatory element (NRE), has a negative effect on the rate of transcription in vivo (110, 121–124) and in vitro (125). Deletion of the NRE also increases the replication rate of the mutant virus 3–5-fold in $CD4^+$ cell lines (124). The NRE contains a number of recognition sequences for cellular transcription factors. The upstream binding factor (USF), originally identified as a cellular factor that binds to the adenovirus major late promoter (126), also binds to a consensus sequence located between nucleotides −173 and −159 of the HIV-1 LTR (W. Haseltine, personal communication). The sequence between −185 and −175 closely resembles the sequences (upstream repres-

sor sequence, URS) that negatively regulate the expression of several yeast genes (127). A protein that is induced early in T-cell activation, designated as nuclear factor of activated T cells (NFAT-1), also binds to a site between nucleotides −254 and −216 in HIV-1 LTR (128). Two binding sites for transcription factor AP1 are located at nucleotides −350 to −345 and −335 to −330 (129). Deletion of AP1 and NFAT-1 sites has no measurable effect on HIV LTR-driven transcription in transient transfection assays. In contrast, deletion of the USF sequence leads to increased LTR-driven transcription as well as higher virus replication in $CD4^+$ T-cell lines (130). It would appear, therefore, that these sequences contain recognition sites for the cellular proteins, which could inhibit transcription initiation.

In addition to the upstream regulatory sequences, HIV promoter contains several protein-binding sites downstream of the RNA start site (Figure 2). A leader binding protein (LBP-1) binds to a region from −17 to +27. LBP-1 specifically recognizes three repeat elements within this region: −3 to +2, +8 to +12, and +18 to +22 (131). In vitro transcription reactions suggest a role for LBP-1 as an activator of initiation. CTF/NF-I, a factor previously shown to bind and activate HSV-1 thymidine kinase and human globin genes promoters, binds to a sequence from +40 to +45 (131). Another protein called untranslated binding protein (UBP-1) has been shown to recognize HIV LTR sequence from −13 to +28 nucleotides (111). Recently, a 43-kDa protein distinct from UBP-1 has been found to bind to nucleotides +37 to +44, a region that overlaps with the transactivation response element (TAR) (R. Gaynor, personal communication).

The precise role of these factors in the basal/induced transcription from HIV promoter remains to be established. Moreover, no information is available at present regarding the number and identity of factors that govern HIV transcription in monocytes, macrophages, and dendritic cells. It is not unreasonable to believe that cell type–specific factors play an important role in HIV gene expression.

Two viral transactivators, Tat and Rev, play extremely important roles in transcriptional and posttranscriptional regulation of HIV gene expression. The details of these viral transactivators are described in sections VA and VB.

D. Assembly and Release of Mature Virus

The selective packaging of viral genomic RNA into particles during assembly is determined by interaction between *cis*-acting sequences present in the RNA and *trans*-acting *gag*-derived nucleocapsid (NC) protein. In all the retroviruses studied so far, the major packaging signal resides in the leader region between U5 and the *gag* gene initiation codon, although sequences located in the *gag* region appear to contribute towards packaging efficiency (132–137). A packaging signal of HIV-1 has also been described. It is located between

the major splice donor and the *gag* initiation codon (138), a situation similar to type-C mammalian retroviruses. Deletion of a 19-base sequence within this region led to 98% reduction in the efficiency of viral RNA packaging. Since this sequence is located downstream of the major splice donor site, it is absent in all subgenomic spliced RNAs, and this could explain a selective encapsidation of genomic RNA. The *trans*-acting component required for viral RNA encapsidation is the RNA-binding nucleocapsid (NC) protein derived from Gag precursor. The NC protein of all retroviruses contain one or two copies of the invariant sequence Cys-X2-Cys-X4-His-X4-Cys, which is reminiscent of the "zinc finger" motif found in many DNA-binding proteins (139). Avian and murine type-C retroviruses with mutations in this sequence produce RNA-deficient, non-infectious virus particles, suggesting the role of this sequence in viral RNA packaging (140–142). Similarly, mutation or deletion of the zinc finger–like sequence of the p9 NC protein of HIV-1 was found to decrease the content of virion-associated genomic RNA to 2–20% of the wild type (143). Furthermore, no infectivity could be detected in the mutant particles, indicating either that these mutants have additional defect(s) for virus replication, or that the inefficient packaging of viral RNA resulted in subthreshold levels of infectious virus. Although it is not known whether retroviral NC proteins require Zn^{2+} for their activity, peptides corresponding to zinc finger–like domains of these proteins do bind Zn^{2+}, and their structures have been determined by spectroscopic methods (144, 145).

Retroviral assembly is a unique process in which the products of *gag* and *pol* are incorporated into virions in the form of their polyprotein precursors during assembly and are proteolytically cleaved during or after budding to form the mature virus particles. One interesting implication of this process is that the individual components of the precursor proteins are active prior to proteolytic cleavage. The process of assembly of HIV particles appears to follow the same pathway as that of type-C retroviruses, with capsid assembly and budding occurring simultaneously. The Gag and Pol proteins are synthesized in the form of their respective precursors $Pr55^{gag}$, NH2-p17-(MA)-p24(CA)-p9(NC)-p7-COOH (146), and $Pr160^{gag\text{-}pol}$, NH2-p17(MA)-p24(CA)-p9(NC)-p7-p10(PR)-p66(RT)-p32(IN)-COOH (147, 148). The p17, p24, and p9 Gag domains as well as p10 protease domain of the Gag and Gag-Pol precursors play important roles in the assembly process. The interactions between the Gag proteins, the plasma membrane, and the virion RNA control the process. The aggregation of $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ molecules under the plasma membrane mediated by $p17^{gag}$ protein initiates the complex process of assembly. In common with other retroviruses (97), the amino terminus of these precursors in HIV is posttranslationally modified by the addition of myristic acid (146–148), which provides a hydrophobic domain supposedly required for interaction with the membrane. As expected

from similar studies carried out on other retroviruses (97), mutation of the amino-terminal glycine of HIV Gag precursor, which abolished myristylation, also resulted in complete disruption of virus assembly (149, 150). However, proteolytic processing of the capsid precursor in these mutants was unaffected, suggesting that the processing of the HIV *gag* gene products occurs before virus assembly. This suggestion is in contrast to results of similar mutational studies carried out on the type-C Mo-MuLv (151) and type-D Mason-Pfizer monkey virus (MPMV) (152).

As discussed earlier, the genomic RNA is brought to the site of assembly by the p9(NC) domain of Gag/Gag-Pol precursor. The p24 Gag, a major core protein, forms a protein shell surrounding the nucleocapsid and therefore determines the tubular shape of the core (153). The interaction between p24 domains of the Gag/Gag-Pol precursors is likely to play an important role in virion assembly. However, no information is available at present about the structure or mechanism of action of p24 in the assembly. The function of p7, a proline-rich carboxy-terminal product of Gag precursor, is not known.

The Gag and Gag-Pol precursors of HIV are cleaved during or after budding to produce individual proteins by the viral-coded protease, p10 (149, 154, 155). This process is responsible for morphological maturation of virions in which the core condenses to form an electron-dense cylindrical structure. Mutations in the HIV protease gene were shown to abolish proteolytic processing of capsid precursor $Pr55^{gag}$ (149, 154, 155). Electron microscopy revealed that assembly and budding steps were not affected in these mutants, but the virions produced resembled immature core particles, suggesting that the protease mediates a postbudding morphological maturation of the core structure (149, 155). Moreover, the mutant particles were non-infectious (154, 155).

As it is clear from the foregoing discussion that proteolytic processing of the polyprotein precursors takes place at a rather late stage of maturation, the question arises as to what prevents the action of the protease domain of Gag-Pol precursor until that stage. It is conceivable that if the enzyme acts only in *trans*, and is only slightly active in the precursor form, then no cleavage should occur until a very high local concentration of precursor is formed in the budding virion. Once the initial cleavage of precursor takes place, the liberated protease may exert its full activity and rapidly process the remaining precursors. An alternative explanation emerges from the deduced structure of avian leukosis-sarcoma virus (156) and HIV (157–159) proteases (for a review see Ref. 160). Both proteins were found to be dimers joined in such a way that the active site is formed by the dimer linkage region. If retroviral proteases are active only as dimers, which is very likely, then such dimers may not be able to form until the precursors have properly aligned themselves as in the late stages of budding. It may be noted that bacterially

expressed HIV protease behaves as a dimer as determined by gel exclusion chromatography (161).

The envelope glycoproteins, synthesized initially as a precursor gp160, are incorporated into virions by a separate pathway. The precursor is cleaved intracellularly, and the products, gp120 and gp41, are inserted into the plasma membrane (162). Cleavage of gp160 is essential for viral infectivity, as mutation of the tryptic-like cleavage site to a chymotryptic site produces non-infectious particles, which can be converted to infectious particles by chymotrypsin treatment (163). The incorporation of envelope glycoproteins into the virions is probably mediated by the interaction between the p17 (MA) domain of Gag precursor and Env protein complex. Direct association between the Gag and Env proteins has also been described for Rous sarcoma virus (165) and murine leukemia virus (166). However, no information is presently available on this aspect in the case of HIV.

It appears then that only the Gag precursor, $Pr55^{gag}$, is required for assembly and release of HIV particles. Such a requirement is consistent with earlier studies on avian and murine retroviruses in which the expression of Gag precursor by itself was found to result in the formation of virus-like particles even in the absence of expression of viral envelope glycoproteins, reverse transcriptase, or viral RNA (167). The expression of *gag* gene alone of HIV in recombinant baculovirus-infected insect cells led to the synthesis, myristoylation, and targeting to the plasma membrane of $Pr55^{gag}$, resulting in the assembly and release of virus-like particles (168). Electron microscopy revealed immature particles, displaying the translucent center and a thick, peripheral electron-dense ring surrounded by an outer lipid membrane. Similarly, expression of the *gag* and *pol* genes of HIV from SV40 late replacement vector in transfected COS cells was shown to direct the assembly and release of virus particles resembling mature HIV particles (169). The particles also contained RNA, but the nature of the RNA was not investigated. It is possible that the RNA packaged was *gag-pol* mRNA containing packaging locus. Virus-like particles were also observed when *gag* and *pol* genes were expressed in a recombinant vaccinia virus system (170). Similarly, the cells coinfected with two recombinant vaccinia viruses, one carrying the HIV-1 *gag* and protease genes and the other the *env* gene, produced HIV-like mature particles, which also contained *gag* mRNA but not *env* mRNA (171). Thus, expression of the Gag precursor protein alone is sufficient for assembly and budding of retroviral particles in general.

Consistent with the role of multimeric Gag precursors in the assembly process, *gag* mutants with deletion or insertion in p24 domain as well as deletion in p17-p24 boundary were reported to behave as *trans*-dominant mutants, interfering with the assembly and/or release of wild-type HIV (172).

IV. ORGANIZATION AND EXPRESSION OF HIV GENES

Molecular cloning (173) and sequencing (174–178) of HIV has revealed that the genomic structure of HIV is more complex than that of conventional type-C retroviruses, including HTLV-I and HTLV-II. In addition to typical retroviral genes, *gag, pol,* and *env,* HIV encodes at least eight other genes (Figure 4; see Section B). The HIV genome displays unprecedented economy in its coding potential as evident from the presence of nine overlapping genes in the 3' half of the genome. For convention, we classify *gag, pol,* and *env* as structural genes, *tat, rev,* and *nef* as regulatory genes, and *vif, vpr, vpu, vpt,* and *tev/tnv* as accessory genes.

A. The Structural Genes

The *gag* gene codes for various structural components of virus particles. It is translated from full-length viral mRNA to produce a polyprotein precursor $Pr55^{gag}$, NH2-p17(MA)-p24(CA)-p9(NC)-p7-COOH. The Gag precursor of HIV differs in some respects from that of other retroviruses. For example, there is no protein interposed between the amino terminus (p17) and the major capsid protein (p24) as there is for most other retroviruses. Also, two small proteins (p9 and p7) are derived from the carboxy terminus of the Gag precursor, unlike one in case of other retroviruses. A detailed scheme of proteolytic processing of the precursor has been proposed (146). Various domains of this precursor play pivotal roles in the assembly and release of virus particles as described earlier (Section IIID).

The *pol* gene of HIV codes for protease, reverse transcriptase, and integrase. In common with other retroviruses, it is translated from the genomic length viral mRNA to produce a polyprotein $Pr160^{gag\text{-}pol}$. The *gag* and *pol* genes overlap by 241 nucleotides and the *pol* gene is in −1 reading frame relative to *gag* reading frame. A ribosomal frameshift, as in the case of avian retroviruses, allows expression of the *pol* gene (179, 180). A frameshift event, which occurs at a low frequency of ~5%, is directed by a short homopolymeric sequence ("slippage sequence") at the *gag-pol* overlap region followed by a hairpin loop structure (for a review see Ref. 97). In the case of HIV, the slippage sequence alone appears to be sufficient to induce ribosomal frameshift (180). This unique strategy of gene expression ensures that the structural (Gag) proteins are made in large amounts and catalytic (Pol) proteins in relatively small amounts.

The precursor is cleaved by the viral protease to produce p10(PR), p66/p51(RT), and p32(IN). The reverse transcriptase polypeptide (p66) is partially cut again, apparently also by the viral protease (181, 182), to remove a 14-kDa polypeptide from the C-terminus. The N-terminal p51, resulting from this cleavage, forms a heterodimer with p66 (183, 184). The purpose of this

additional cleavage is presently unclear. The fate and a possible function of the C-terminal 14-kDa protein are also not understood, though there is some evidence that it exists as a separate protein having RNase H activity (185).

The HIV reverse transcriptase, in common with other retroviral reverse transcriptases, has polymerase as well as RNase H activities. Mapping studies have shown that the N-terminal two-thirds of the molecule constitutes the polymerase domain, but no distinct domain could be assigned the RNase H function (186, 187). It appears that the optimum folding of the RNase H domain requires the entire protein sequence. Alternatively, discontinuous regions distributed throughout the molecule may directly participate in the formation of RNase H active site. Such a distribution is in contrast to the case in Molony-MuLV reverse transcriptase, in which two distinct regions carry out these two enzymatic activities (188). The RNase H activity plays a crucial role in proviral DNA synthesis. It carries out endo- as well as exo-nucleolytic hydrolysis of RNA from RNA:DNA hybrids with limit digestion products ranging in size from 5 to 20 nucleotides. Interestingly, a polypurine tract immediately upstream of the U3 region is left intact in order to create a primer for the synthesis of the plus strand of DNA (97).

Because of the lack of proofreading function, all reverse transcriptases (RTs) are notoriously error-prone (97). HIV RT seems to be significantly more error-prone than other RTs, with frequency of misincorporation ranging from 1:1700 to 1:4000 (189, 190), which appears to partly account for the high mutation rate of HIV in vivo.

The HIV-1 integrase has been expressed and purified from *Escherichia coli*. The purified protein has been shown to cleave adjacent to a conserved CA dinucleotide in a synthetic oligonucleotide substrate, resulting in the removal of two nucleotides from the 3' ends of both the U5 plus strand and the U3 minus strand (191). Thus, the purified protein simulates some of the steps in retroviral integration ascribed to integrase function.

The envelope glycoproteins of HIV are synthesized initially as an 88-kDa precursor inserted into the rough endoplasmic reticulum, where the addition of high-mannose N-linked carbohydrate chains as well as folding into an appropriate tertiary structure takes place (192, 193). The carbohydrate chains are terminally modified in the Golgi complex. The gp160 precursor is cleaved in a nonlysosomal acidic compartment by some cellular protease, and the mature envelope proteins (gp120 and gp41) are transported to the cell surface (162, 193). Only a small amount (5–15%) of gp160 is cleaved to produce gp120 and gp41, while most of the uncleaved gp160 is delivered to lysosomes, where it is degraded (162). It is not clear why most of the gp160 is routed to lysosomes, while gp120 and gp41 are transported to the cell surface. The possibility that such routing is a general feature of retroviral glycoprotein maturation is suggested by similar results obtained in case of Rous sarcoma

virus (194–196). In any case, it explains the requirement of intracellular cleavage of gp160 for the production of infectious particles (163). In common with other retroviruses (197), the envelope glycoproteins of HIV also exist in oligomeric forms. It has been shown that the precursor gp160 forms stable homodimers that in turn assemble into higher-order structures, most likely representing tetramers composed of two dimers each. The molecule retains its oligomeric form after cleavage to gp120/gp41 (198). It appears that the extracellular domains of adjoining gp41 mediate oligomerization of gp160. Consistent with this, native gp41 in virions appears primarily to be a tetramer (199). Though noncovalently associated, these complexes were stable when boiled in the presence of a low concentration of SDS. Using various cross-linking reagents, another study has demonstrated tetrameric forms of gp160 and gp41 (200). The oligomeric form of gp160 is likely to be important for intracellular transport and stability. Moreover, it may increase the avidity of virus binding.

B. The Regulatory Genes

The *tat* gene is encoded by two exons, one preceding the *env* gene coding for 76 amino acids and the other within the *env* gene coding for 12 amino acids (201). It is translated from various multiply spliced mRNAs (discussed in detail in Section VA). A predominant form of Tat in the infected cells is an 86-amino-acid long (16-kDa) protein derived from two-exon mRNAs (202, 203). Because of a stop codon immediately following the splice donor site of the first *tat*-coding exon, a minor form of 72-amino-acid (14 kDa) Tat is expressed from additional unspliced one-exon mRNAs. The Tat protein is a transactivator of LTR-directed gene expression (201, 204). It is absolutely essential for viral replication, as the Tat-defective mutants do not make virus unless Tat protein is provided in *trans* (205, 206). The N-terminal 72 amino acids of the Tat protein, encoded by the first exon, appear sufficient for full transactivation of HIV LTR-specific gene expression (204). Furthermore, mutational analysis has shown that the amino-terminal 58 amino acids of Tat are sufficient for transactivation, although with reduced activity (207, 208).

Three important functional domains have been identified in Tat (Figure 3). The acidic amino-terminal region has been proposed to have a periodic arrangement of acidic, polar, and hydrophobic residues consistent with an amphipathic α-helix (209), a feature reminiscent of activation domains of many transcription factors (210). Site-directed mutagenesis has shown that conservative changes in these acidic amino acids are well tolerated, whereas nonconservative changes markedly reduce Tat activity. Whether the acidic region of Tat folds into an α-helix and serves as an activation domain requires further study. A cluster of seven cysteine residues, highly conserved among divergent isolates of HIV-1, HIV-2, and SIV Tat proteins, constitutes the

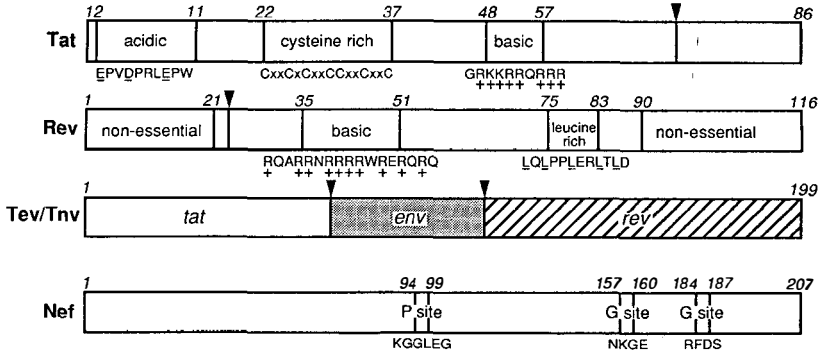

Figure 3 Schematic representation of the functional domains of HIV-1 regulatory proteins. Various numbers refer to the amino acid positions. Inverted triangles separate the regions contributed by adjacent exons. See text for details.

second domain (Figure 3). Mutation of all but one (residue 31) of these cysteine residues destroys activity (211–213). It has been proposed that Tat forms a metal-linked dimer with metal ions bridging cysteine-rich regions from each monomer (214). However, the existence of Tat dimers in vivo and their potential role in Tat activity are yet to be established.

A stretch of basic amino acids, from 49 to 57, constitutes the third domain and is required for nuclear localization (213, 215). Mutations within this region yield a cytoplasmic Tat protein, which is nonfunctional. The nuclear signal motif, GRKKR (amino acids 48 to 52), is sufficient to induce the nuclear localization of the normally cytoplasmic β-galactosidase gene product when introduced at the amino terminus (213). Interestingly, Tat has been shown to be predominantly localized to the nucleolus (213, 216). However, a sequence required for this subnuclear localization has not been identified. By analogy with the Rev protein, which is also a predominantly nucleolar protein, it is conceivable that the nucleolar targeting signal may be a bigger sequence extending on both the sides of the nuclear targeting signal.

Rev is the second regulatory protein of HIV-1 that is essential for viral replication (217, 218). It is a 19-kDa protein (116 amino acids) predominantly located in the nucleolus (219). Rev is expressed from two coding exons contained in a variety of multiply spliced mRNAs (211, 220). Although it has been shown to be phosphorylated at serine residues (221), phosphorylation is not essential for its transactivation function since serine substitution mutants that are not phosphorylated are fully active (222, 223). The amino-terminal 20 amino acids as well as the carboxy-terminal 25 amino acids are dispensable for Rev activity (222–224). Two distinct domains have been identified in Rev (Figure 3). A stretch of basic amino acids appears to be important, since

deletions within it destroy the activity of Rev (223, 225). The motif NRRRRW confers nuclear localization (224, 226, 227), and it can also substitute for the nuclear localization domain of Tat (228). Removal of the amino-terminal portion of the basic region, which permits concentration of Rev in the nucleus but excludes it from the nucleolus, results in inactive proteins (224, 227, 228), indicating that nucleolar localization is crucial for Rev activity. Additionally, the basic sequence is responsible for interaction with the Rev RNA target sequence, RRE (229).

A leucine-rich sequence, LQLPPLERLTLD, constitutes the second important domain of Rev. Substitutions of the underlined leucine residues yield inactive Rev proteins that act as *trans*-dominant inhibitors of wild-type Rev (223, 230). The leucine-rich sequence has been thought to act as an "activation" domain. Thus, Rev appears to have a modular structure, as in the case of many eukaryotic transactivators (210), with a basic region acting as a "specificity" domain responsible for interaction with RRE-containing transcripts and an "activation" domain responsible for inducing nuclear export of such transcripts as a consequence of the binding event.

The *nef* gene is contained in a single open reading frame at the 3' end of genome overlapping the *env* gene and the 3' LTR. A number of multiply spliced mRNAs encode the Nef protein (Figure 4). Though the *nef* open reading frame is present in all HIV and SIV isolates, it frequently contains premature termination codons in virus strains that have been extensively passaged in tissue culture. Moreover, many lentiviruses such as visna virus, CAEV, and EIAV do not contain sequences similar to that of the *nef* gene. Nef is a 25–27-kDa cytoplasmic protein (231, 232). It is myristoylated at the penultimate glycine residue of the amino terminus, and is found in association with the inner plasma membrane presumably through this fatty acyl moiety (233). A free nonmyristoylated Nef has also been detected in the cytoplasm. Nef protein has also been found to be secreted from hamster kidney cells infected with recombinant vaccinia virus expressing *nef* gene (234).

Some features of Nef, such as its location at the inner face of plasma membrane and sequence similarity with guanine nucleotide–binding proteins (G proteins), led to the prediction that Nef is involved in signal transduction similar to the G proteins (235). The idea acquired momentum when it was reported that partially purified Nef protein expressed in *E. coli* indeed exhibited GTP-binding, and GTPase and autokinase activity (236). It was further supported by reports that Nef down-regulates HIV LTR-directed gene expression as well as viral replication (as discussed in section VC). However, many groups have since reported results that failed to support these claims. Recently, Nef protein from BH10 and LAV-1 strains of HIV-1 expressed in *E. coli* and purified to apparent homogeneity was found not to have GTP-binding or GTPase activity (237). Nevertheless, Nef protein does have sequence homol-

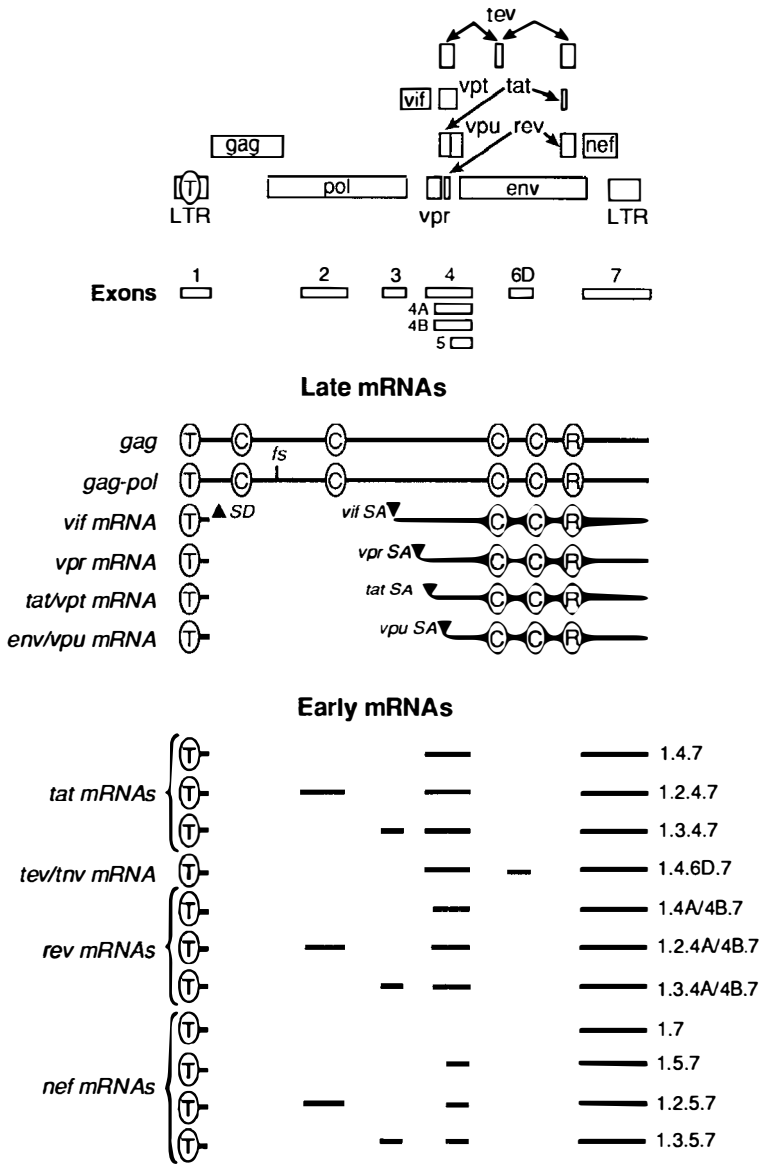

Figure 4 The genomic organization of HIV-1 and the pattern of splicing. The exons are numbered essentially as described by Schwartz et al (220). T, *trans*-activation response (TAR) element; C, *cis*-acting repressor sequences (CRS); R, Rev response element (RRE). The major splice donor (SD) site is depicted by a triangle, and various splice acceptor (SA) sites are depicted by inverted triangles. fs represents the site of ribosomal frameshift during the translation of *gag-pol* mRNA. Combinatorial splicing of various exons in early mRNAs is shown on the right. See text for details.

ogy with GTP binding sites of G proteins (Figure 3). This includes a P site with a consensus sequence of GXXXXGK (KGGLEG in the case of Nef) and two G sites with consensus sequences NKXD and WRFD (NKGE and RFDS in the case of Nef). Nef mutants at the putative P site were found to be increasingly exported from the cell and had decreased phosphorylation. Deletion of the RFDS sequence resulted in a dramatic reduction in the half-life of Nef (234).

C. The Accessory Genes

One common characteristic of the accessory genes is that many of them are defective in HIV strains that are extensively passaged in tissue culture, probably because they are dispensable and therefore are selected against during in vitro growth. However, they are likely to serve some important role during in vivo growth of virus and consequently are highly conserved in natural isolates.

A novel protein of 28 kDa designated as Tev (238) or Tnv (239) has recently been described in HIV-1-infected cells. It contains 72 amino acids from the first exon of Tat at its amino terminus, 38 amino acids from the *env* region in the middle, and 91 amino acids from the second exon of *rev* at its carboxy terminus (Figure 3). $p28^{tev}$ has both Tat and Rev activities, and its expression is temporally regulated as in the case of Tat and Rev. Its potential regulatory role, however, is yet to be established.

The *vif* gene encodes a 23-kDa cytoplasmic protein designated as virion infectivity factor (240, 241). The *vif* mRNA may additionally code for Vpu and one-exon Tat (Figure 4) and therefore act as a polycistronic mRNA. *Vif*-defective mutants are not compromised in the level of intracellular viral proteins and extracellular virus (242, 243). However, although the mutant viruses are morphologically and biochemically indistinguishable from wild-type, they exhibit a profound reduction in their efficiency to infect many $CD4^+$ cell lines (242, 243). The magnitude of reduction varies depending on the cell line. The *vif* mutation predominantly affects cell-free virus transmission, with little effect on the cell-to-cell mode of viral transmission. That the *vif* gene product exerts its effect in *trans* is evident by complementation of *vif* mutant viruses by cotransfection with *vif* expression plasmid (242). Considering the fact that Vif is not a virion-associated protein, it is difficult to explain its profound effect on virus infectivity. This protein may somehow modify some component of the virus particles in a way that makes them more infectious. The precise mechanism of action, however, remains to be established.

The *vpr* gene encodes a 15-kDa protein (96 amino acids) in most strains of HIV-1 (244), although the open reading frame is often truncated in viral strains extensively passaged in tissue culture. The *vpr* open reading frame is also present in HIV-2 isolates and in most but not all isolates of SIV. The Vpr

protein contains numerous hydrophilic regions and several regions of clustered positive charges. It has recently been shown to be present in mature virus particles in multiple copies (245). Since Vpr is not known to be part of any larger precursor such as Gag or Gag-Pol, it must be incorporated independently into virions. Consistent with this possibility, Vpr was found to be incorporated in *trans* as shown by cotransfection of *vpr* expression plasmid and *vpr*$^-$ provirus (E. Cohen and W. Haseltine, personal communication). Interestingly, Vpr is the first regulatory protein of any retrovirus found to be associated with virus particles. Other regulatory proteins, such as Tat, Rev, Nef, Vif, and Vpu, are not virion associated.

Vpr accelerates the replication and cytopathic effect of HIV-1 in CD4$^+$ T-cells, with the most pronounced effect exerted early in infection (246, 247). The accelerating effect of Vpr on viral replication has been demonstrated not only in various T-cell lines but also in primary cultures of T-cells, monocytes, and macrophages. Vpr stimulates expression of reporter genes linked to HIV-1 LTR by about threefold (248); this increase is reflected by a proportionate increase in the steady state level of HIV LTR-directed RNA. Vpr-responsive *cis*-acting sequences are yet to be identified. Interestingly, Vpr and Tat appear to have a synergistic effect on HIV LTR-driven gene expression. In contrast to Tat, Vpr stimulates gene expression from a variety of viral and cellular promoters, such as LTRs from HTLV-1, Rous sarcoma virus, and the murine retrovirus (SL3-3) as well as SV40 early promoter and IL-2 promoter. The precise mode of action of Vpr is yet to be established. Some of the possibilities include stimulation of RNA initiation and stabilization of RNA. The presence of Vpr protein in virions suggests that it may function at some early steps in the virus replication cycle, such as the formation and integration of provirus or the initial rate of transcription from the provirus.

The *vpu* gene encodes a 15–20-kDa (81 amino acids) protein (249–251). It is expressed from a polycistronic mRNA that also encodes the envelope protein. The *vpu* reading frame lacks the initiation codon in many HIV-1 strains passaged extensively in tissue culture (E. Cohen and W. Haseltine; G. Pavlakis, personal communications). The *vpu* gene is unique to HIV-1 as it is absent in HIV-2 and in most SIV isolates. The Vpu protein has strong hydrophobic and hydrophilic regions at its amino- and carboxy-terminal regions, respectively. It is also phosphorylated at one or more serine residues (250). Although the *vpu* open reading frame is constant in length among different isolates, there is large variation in the apparent molecular weights of the Vpu proteins because of the anomalous electrophoretic mobilities on denaturing gels. For example, the apparent molecular weights determined on denaturing gels for ELI and IIIB Vpu are 15 and 20 kDa, respectively (252). This observation suggests that the protein is highly ordered, or small changes

in charge distribution dramatically alter the electrophoretic mobility of the protein. Vpu is a cytoplasmic protein and is not found in the virions.

Vpu protein acts in a subtle way. While the total amount of viral protein made in infected cultures is the same for vpu^+ and vpu^- viruses, the latter show a decrease in the amount of extracellular virus, which correlates with intracellular accumulation of viral proteins, and more rapid formation of syncytia and cytopathic effects (250, 252, 253). Additionally, T-cells infected with vpu^- virus show numerous virus particles within intracytoplasmic vesicles (253). This analysis has suggested a role of Vpu in facilitating assembly and/or release of virus particles. However, the precise mode of action is not known.

The *vpx* gene, present only in HIV-2 and SIV but not in HIV-1, encodes a 16-kDa virion-associated protein (254--256). Although it appears to be dispensable for viral replication in lymphocytic cell lines (257), vpx^- mutants are less efficient in infecting primary lymphocytes (258). The *vpx* gene and its product are yet to be characterized.

An open reading frame designated as *vpt* has been detected in all HIV-1 isolates (259) as well as in closely related chimpanzee isolates (260), but not in HIV-2 and SIV isolates. This open reading frame does not contain an AUG initiation codon. However, a consensus -1 frameshift sequence exists near the 5' end that may permit expression of this open reading frame by ribosomes initiated at the *tat* AUG codon. Consistent with this possibility, a 17-kDa Tat-T fusion protein was detected upon in vitro translation of RNA transcripts derived from this region (261). The fusion protein is recognized by antisera raised against the amino terminus of Tat and peptides derived from this predicted sequence at the carboxy terminus of T. Furthermore, elimination of the consensus frameshift sequence or the *tat* AUG codon precludes synthesis of Vpt. This fusion protein has neither Tat nor Rev activity, and does not interfere in these activities. The expression and function of Vpt in natural infection have not been established. The protein has not been detected in HIV-1-infected cells, and antibodies to T-specific peptides have not been found in HIV-1-infected people (261). Nonetheless, the remarkable conservation of the *vpt* reading frame and the consensus frameshift sequences among diverse HIV-1 isolates suggest an important role for *vpt* in HIV infection.

Interestingly, an open reading frame has been detected on the plus strand of HIV-1 DNA and has been suggested to encode a protein (262). Recently, three RNAs of 1.6, 1.1, and 1.0 kb have been detected on Northern blot of poly(A)$^+$ RNA derived from HIV-1-infected H9 cells using HIV plus strand transcript as a probe (263). The expression of these RNAs was restricted to the early phase of infection. However, the significance of this observation awaits further studies.

D. Splicing Pattern

HIV has evolved an unprecedented complexity in splicing, giving rise to a multitude of spliced mRNAs from a single primary transcript. The 3' half of the genome has a very intricate layout of multiple, often overlapping open reading frames (Figure 4). Most replication-competent retroviruses express two types of mRNAs: the genomic RNA, which also encodes the Gag and Gag-Pol precursors, and singly spliced subgenomic RNA, which encodes the Env precursor. In addition to these two species of mRNAs, HIV codes for a variety of multiply spliced mRNAs, some of which produce the regulatory and accessory proteins.

The organization of various exons and the pattern of splicing is depicted in Figure 4. Multiply spliced mRNA species have been cloned using polymerase chain reaction (PCR) amplification, and some of the clones analyzed functionally (220). Exon 1, derived from the 5' region of the genome (R, U5, and a small leader sequence) is common for all HIV RNAs. Similarly, exon 7 is present at the 3' end of most of the HIV RNAs. The regulatory proteins (Tat, Rev, and Nef) are each produced from at least three mRNAs differing in the presence or absence of one of the noncoding exons 2 and 3. For example, Tat is encoded by mRNAs generated by splicing of the central exon 4 (264) to exon 1 directly or via exon 2 or exon 3 (220). Two splice acceptor sites have recently been identified between the *tat* and the *rev* AUGs, which are used for *rev* expression (220). The corresponding exons are designated as 4A and 4B, and these splice sites are located 15 and 9 nucleotides upstream of the *rev* AUG, respectively. Thus, utilization of either splice acceptor 4A or 4B allows expression of *rev* but not *tat*. These two exons are spliced to exon 1 either directly or via exon 2 or exon 3, generating six different *rev* mRNAs (220). A splice site between the *rev* and *tat* AUGs has also been reported earlier by S1 nuclease analysis (211). Likewise, three *nef* mRNAs are generated by splicing of exon 5 (220, 264) to exon 1 directly or via exon 2 or exon 3 (220). Exons 2 and 3 are mutually exclusive within the multiply spliced mRNAs. There are conflicting reports as to whether exon 1 can be directly spliced onto exon 7 (220, 265). The presence or absence of small noncoding exons 2 and 3 plays no significant role in the expression of the multiply spliced mRNAs (220). Functional studies have shown that *tat* mRNAs, although polycistronic in nature, produce very low levels of Rev and Nef, while *rev* mRNAs produce high levels of both Rev and Nef proteins (220). It has also been shown that *nef* is the most abundant multiply spliced mRNA species in HIV-1-infected cells, independent of the strain of the virus and the cell type, and is seen in both chronic and short-term infections. Relatively, *tat* mRNA is expressed at extremely low levels, while *rev* mRNA is present at intermediate levels (265).

Another small exon located within the *env* gene, designated as 6D, has recently been identified. This exon is used to generate a hybrid protein Tev (238). Two other exons, 7A and 7B, have also been identified (Figure 4), with splice acceptors located 28 and 24 nucleotides upstream of exon 7, respectively (220). These exons are rarely used, however, and the presumptive mRNAs have not been functionally analyzed. Many additional multiply spliced mRNAs with different combinatorial association of various small exons, which could enormously expand and diversify the coding potential of HIV, have been detected using PCR. However, the functional utilization of such mRNAs and the significance of the protein products, many of which are expected to be variants, remain to be demonstrated. One common feature of all the multiply spliced mRNAs is the removal of RRE, and therefore nuclear export of these RNAs is independent of Rev. It is clear from the foregoing discussion that there is a great deal of structural and functional redundancy in HIV mRNAs, the significance of which is beyond our comprehension at present. The incorporation of additional splice signals and generation of many alternatively spliced mRNAs has been made possible by the acquisition of regulatory factors such as Rev, which ensures the utilization of appropriate quantities of unspliced/singly spliced structural mRNAs.

Proteins such as Vif, Vpr, Vpt, Vpu, and Env could be made from intermediate sized, singly or doubly spliced mRNAs. For example, a single splicing event between the splice donor site of exon 1 and splice acceptor site of exon 2 can generate *vif* mRNA. It is possible that Vpr as well as one-exon Tat can also be made from this RNA. *Vpr* mRNA is probably generated by splicing of exon 1 to the splice acceptor site of exon 3. Some of these intermediate size mRNAs are well characterized. Three different categories of *env/vpu* mRNAs utilizing exon 4A, 4B, or 5 have been demonstrated by PCR amplification and cloning (266). In each category the first exon was found to be spliced to the 3' end long exon, either directly or through exon 2 or exon 3, thereby generating a total of nine mRNA species. All the *env/vpu* mRNAs expressed both Env and Vpu proteins and therefore are functionally bicistronic. Translation of these mRNAs is consistent with the "scanning model" (267) where Env is produced by leaky scanning from mRNAs that contain *env* in the second or third reading frame. This is apparent as the AUG codons of the upstream open reading frames (*vpu* and/or *rev*) are not in a favorable context. Similarly, a group of three mRNAs coding for the one-exon Tat and Vpt have been shown to result from utilization of the splice acceptor site of exon 4 with or without exon 2 or 3 (266). This mRNA contains several downstream open reading frames (*rev*, *vpu*, and *env*). The *tat* reading frame, however, owing to the favorable context of its AUG codon, appears to block the expression of downstream open reading frames.

V. NOVEL REGULATORY PATHWAYS IN HIV GENE EXPRESSION

A. Transcriptional and Posttranscriptional Activation by Tat/TAR

Tat is absolutely essential for HIV transcription and consequently for viral replication. The *cis*-acting Tat-responsive element (TAR) was originally localized to nucleotides +1 to +80 within the viral LTR (268). Subsequent studies have further mapped its 3' boundary to +44 (269–271). The location of TAR within the transcribed region distinguishes it from *cis*-acting sequences of various cellular and viral transcriptional activators, which are almost always located upstream of the transcription initiation site. Because of its location, TAR is present at 5' end of all HIV RNAs. The activity of TAR is strictly position- and orientation-dependent. It functions only in its native orientation and when positioned immediately downstream of the site of transcription initiation (266, 270–274). Although it is not completely ruled out that TAR acts as DNA, much direct and indirect evidence strongly suggests that it acts in form of an RNA element. The TAR RNA assumes a stable stem-loop structure in vitro (Figure 5), as determined by nuclease mapping (273). Structural features of TAR that are important for Tat-mediated transactivation include the primary sequence in the loop (nucleotides 31 to 34), the 3-nucleotide bulge (nucleotides 23 to 25), and an intact stem (269–271, 275–277). Accordingly, nucleotide substitutions in the loop as well as the bulge dramatically reduce TAR activity, whereas mutations in the stem are tolerated as long as compensatory mutations are made to restore stem structure. Through use of a novel mutagenesis approach directed towards changing only the secondary structure of TAR, evidence has been provided in favor of TAR acting as RNA. The same study has also shown that the mutant transcripts that only transiently form a native TAR RNA structure, which is not maintained in the mature RNA, are efficiently transactivated by Tat, suggesting that TAR is recognized as a nascent RNA (278).

Purified Tat has been shown to interact with TAR RNA in vitro (279, 280). Use of mutant TAR demonstrated that the bulge was important for both interaction of Tat with TAR RNA as well as Tat-mediated transactivation. In contrast, mutations in the loop and the stem, which reduce Tat-mediated transactivation, had no effect on Tat binding (280). Therefore, direct binding of Tat to TAR appears to be important but not sufficient for transactivation. In conflict with this conclusion are recent studies that showed that Tat can activate transcription by direct binding to nascent RNA, and that the sole function of TAR may be to bring Tat to the vicinity of the promoter by virtue of providing a Tat-binding site. A Tat-Rev fusion protein has been reported to

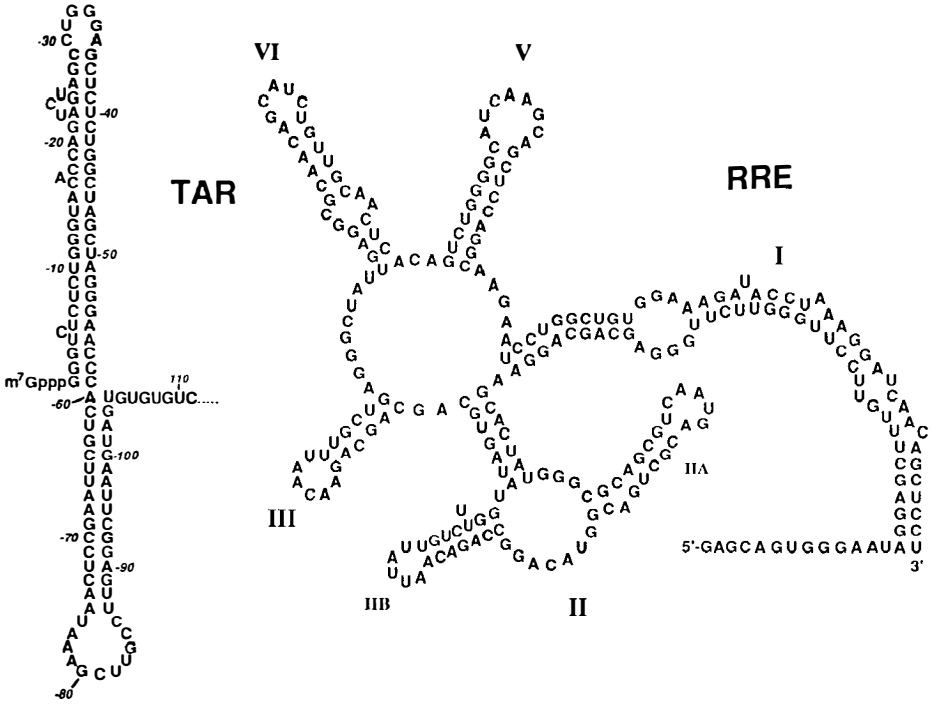

Figure 5 The nucleotide sequence and predicted secondary structure of *trans*-activation response (TAR) element and Rev response element (RRE).

activate transcription from an HIV-1 promoter derivative, in which TAR is replaced by the Rev response element (RRE) (281). Similarly, Tat fused to the coat protein of bacteriophage MS2 can transactivate an HIV-1 LTR that contains a phage operator sequence in place of TAR (282). The use of fusion protein also identified a stretch of basic amino acids (from 49 to 57) as the RNA-binding domain and the N-terminal part comprising α-helical and cysteine-rich regions as activation domains (282).

In the above reports, RNA-binding proteins were fused to Tat. Yet another study has shown that a Tat-Jun fusion protein, which contains the DNA-binding domain of v-Jun, could transactivate the reporter gene containing four copies of the AP-1/Jun-binding sites inserted in the TAR region (283). The insertion of AP-1 sites in TAR was shown to inactivate TAR function. Thus, the promoter-proximal positioning of Tat, either through RNA- or DNA-binding mechanisms, is essential for transactivation. The latter study (283) also showed that the deletion of NFκB and Sp1 binding sites from the HIV promoter abrogates Tat-mediated transactivation, suggesting that once

brought into the vicinity of the promoter, Tat may interact with transcription factor(s) bound at the NFκB/Sp1 region of the HIV promoter to stimulate transcription initiation and stabilize elongation complexes.

Cellular factors have been postulated to play a role in Tat-mediated transactivation. This suggestion stems from the observation that HIV-1 expression as well as HIV-1 LTR–directed heterologous gene expression are greatly reduced in rodent cells as compared to human cells. Through use of human–Chinese hamster ovary (CHO) hybrid cell clones, the presence of human chromosome 12 was found to be required for Tat-mediated transactivation (284). Several proteins present in nuclear extract of HeLa cells have also been shown to bind TAR RNA in vitro (285–287). A 68-kDa protein has been identified by UV cross-linking to TAR RNA, and the binding affinity of several TAR mutants for this protein was found to correlate with the ability of these mutants to support Tat-mediated transactivation (287). Similarly, a protein that interacts with Tat has been described. A cDNA coding for a 45-kDa nuclear protein, designated as Tat-binding protein-1 (TBP-1), has been cloned by screening a λ-gt11 expression library with Tat as a probe (288). The potential role of these Tat- or TAR-binding proteins in Tat-mediated transactivation needs further investigation. The fact that reporter constructs in which TAR is replaced by the recognition sequences of the RNA/DNA-binding proteins are efficiently transactivated by the Tat fusion proteins (281–283) indicates that transactivation by Tat can occur in the absence of cellular TAR DNA- and RNA-binding proteins.

Tat has also been proposed to act as an antiterminator of RNA elongation (274). When the HIV-1 LTR was placed on a replicating SV40 vector, the primary effect of Tat was reported to be permitting RNA polymerase II to continue to elongate past a strong termination site located approximately 60 nucleotides from the site of RNA initiation. Tat did not exert a pronounced effect on the rate of initiation in these experiments. A separate study (289) confirmed that Tat reduces polarity due to premature transcriptional termination in downstream sequences, but also showed that Tat stimulates the rate of transcription of the first 25 bp region by approximately 15-fold. It is difficult to reconcile the termination suppression model with the observations that removal of TAR does not increase the basal activity of HIV LTR, and that the TAR does not exert a negative effect on heterologous promoters when placed downstream of initiation site.

Most eukaryotic transactivators interact, directly or indirectly, through another protein, with their cognate DNA sequences in the vicinity of the promoter, and the resultant secondary interaction with the ubiquitous transcriptional factors (such as TF-IID) supposedly stimulates the rate of transcription. Tat may be considered as a unique transcription factor that accomplishes transcriptional stimulation by interacting with the nascent tran-

script. The following scenario could be envisioned. The basal activity of the HIV-1 LTR results in low-level initiation of viral RNA, and synthesis of a small amount of Tat protein. The newly made Tat protein binds to the TAR sequence at the 5' end of the RNA and interacts, directly or indirectly, with the transcription machinery to promote RNA initiation and/or efficiency of elongation of the initiated transcripts. Thus, TAR can be regarded as an "RNA enhancer" (290). The interaction of Tat with TAR RNA may recruit certain transcription factors and consequently facilitate assembly of the transcription initiation complexes at the promoter. However, since TAR acts as an RNA element, it shows orientation dependence due to the polarity of transcription and it functions only downstream of the site of initiation.

Another effect of this multifaceted protein has been reported at the post-transcriptional level. Tat dramatically induces the expression of viral proteins as well as heterologous proteins directed by HIV-1 LTR, attributable both to the low basal activity of the HIV-1 LTR in the absence of Tat and to a high induced level of protein synthesis in the presence of Tat. The extent to which increase in RNA concentration or increase in the efficiency of utilization of the transcribed RNA accounts for the dramatic increases in Tat-induced HIV-1 LTR-directed protein synthesis is controversial and appears to depend upon the experimental system used to measure the effect. Though all investigators concur that the concentration of all HIV-1 LTR-directed RNA rises in the presence of Tat, some investigators report that the increase in RNA is not enough to account for the total increase in the protein synthesis. For example, the Tat-induced 100-fold increase in gene expression could be divided into a 20-fold increase in mRNA synthesis and a 5-fold increase in the amount of protein synthesized per mRNA. A bimodal action of Tat has indeed been proposed (291). More direct evidence in favor of posttranscriptional activation by Tat comes from the study in which coinjection of purified Tat protein and in vitro synthesized TAR-containing CAT mRNA into the nuclei of *Xenopus* oocytes resulted in a substantial increase in CAT protein synthesis (292). Furthermore, this activation was not inhibited by transcriptional inhibitors, and it occurred only when the RNA was injected into the nucleus and not into the cytoplasm. It was proposed that in the presence of Tat, the TAR-containing RNA is either chemically modified or becomes associated in the nucleus with cellular factors and/or with Tat itself, resulting in enhanced translatability. The relevance of these observations to mammalian systems is not clear, however, especially in light of the observation that no transactivation is observed when purified Tat protein is added to mammalian cells in the presence of transcription inhibitors (293).

The observation that mutations that prevent nucleolar localization abrogate Tat activity raises the possibility that the nucleolus may be involved in Tat regulation of gene expression. It may be recalled that 5S RNA, made in the

nucleoplasm, is transported into the nucleolus from which it is rapidly exported into the cytoplasm. The nucleolus also serves as the site of assembly and rapid transport of ribosomes from the nucleus to the cytoplasm. It is conceivable that Tat protein makes use of this cellular RNA transport process to achieve efficient export of TAR-containing RNA from the nucleus.

Until recently, Tat had been thought to be highly specific for the HIV LTR, unlike the HTLV transactivator Tax, which has been shown to transactivate a number of cellular gene promoters. It has now been reported, however, that Tat can transactivate a heterologous promoter, the JC virus late promoter (294). Transactivation of this promoter appears to be cell-type specific as it was observed only in glial cells, but not in T-cells or fibroblasts, indicating a possible involvement of cell-type specific factors. This activity of Tat may contribute to a neurological disease, progressive multifocal leukoencephalopathy (PML), by reactivation of latent JC virus.

B. Regulation of Splicing/Transport of mRNA by Rev/RRE

HIV has a remarkable property of differentially regulating the expression of structural vs regulatory genes in a temporal manner. In the early phase, ~2 kb mRNAs encoding the regulatory proteins Tat, Rev, and Nef predominate. A marked shift takes place in the late phase, leading to the predominant expression of unspliced (~9 kb) and singly spliced (~4 kb) mRNAs encoding structural proteins including Gag, Pol, and Env precursors (295, 296) and many of the accessory proteins. The shift from early to late phase is mediated by the Rev protein (217, 218, 297, 298; for reviews see Refs. 295, 299). Transactivation of viral structural gene expression by Rev requires *cis*-acting RNA sequence designated as Rev response element (RRE), which is located in the *env* gene, and is removed by splicing to generate the small regulatory mRNAs (300–302). The RRE only functions in the sense orientation and within a transcription unit (300, 303). The RRE has been predicted to form a complex secondary structure (301) as shown in Figure 5. The central stem I is formed by complementary sequences at 5' and 3' termini of RRE. The intervening stem-loop structures are designated in a clockwise fashion stem-loops II, III, IV, and V, respectively. The stem-loop II, a hammerhead structure, consists of two smaller stem-loop structures, IIA and IIB. The minimal RRE required for biological activity is approximately 200 nucleotides as determined by deletion mutagenesis (304, 305). Within this structure, the stem-loop II appears to be the most important element for in vivo activity (304–306).

A specific and high-affinity interaction between purified Rev protein and RRE has been demonstrated in vitro (304–310). A single hairpin loop structure, stem-loop II, was found to be a primary determinant for Rev binding in vitro and Rev response in vivo (305, 306). Maintenance of secondary struc-

ture, rather than primary nucleotide sequence alone, appears to be necessary for Rev-RRE interaction (305). A 90-nucleotide subfragment of RRE at 5' end is sufficient for Rev binding (310). Interestingly, the same subfragment also binds specifically and predominantly to a seemingly ubiquitous mammalian nuclear factor designated as NF_{RRE} (Nuclear Factor, RRE-binding). Furthermore, this ~56-kDa protein is the only detectable cellular factor that binds to the whole RRE (310a). The significance of this observation awaits further study. It may be noted, however, that the 90-nucleotide sequence is insufficient to confer Rev response in vivo, indicating that the RRE sequences 3' to this element are required for translating binding events into function (310a). The Rev protein has been suggested to bind RRE as a multimer on the basis of an intermediate gel shift of the labelled RRE in the presence of both the wild-type and a truncated Rev protein as compared to the extents of gel shift obtained with the two proteins separately (229). It is also consistent with the stoichiometric data obtained using a nitrocellulose filter-binding assay (307) as well as the observation of four distinct complexes detected with a smaller fragment of RRE as a probe in the gel shift assay (Y. Vaishnav and F. Wong-Staal, unpublished). Interestingly, a mutant Rev protein, previously shown to function as a *trans*-dominant inhibitor of Rev function (223), was found to bind RRE as a multimer to a similar extent as wild-type Rev (229). The results suggest the existence of cellular factor(s) that may interact with Rev or Rev/RRE complex, and this interaction may be important for transactivation. The *trans*-dominant Rev mutants probably lack the capacity to interact with these cellular factor(s) and therefore interfere with wild-type Rev by competing with them for binding to RRE.

Expression of unspliced and singly spliced RNA into protein is prevented by *cis*-acting repressive sequences (CRS) present in *gag, pol,* and *env* genes of the HIV genome (300). CRS elements repress expression when introduced into mRNA of heterologous genes such as CAT, but the repression is overcome by introducing HIV-1 RRE sequence in *cis* and supplying Rev in *trans*.

Rev is a posttranscriptional regulator of gene expression as it affects the fate of the completed RNA transcript. Rev was originally identified as an antirepressor of translation *(art)* because mutation in *rev* gene resulted in greatly diminished viral structural protein synthesis without affecting viral transcription (218). The suggestion that Rev might regulate the splicing process came from the observation that rev^- mutant proviruses are defective in the expression of unspliced and singly spliced mRNAs, encoding structural proteins in the cytoplasm, and hence the term *trans*-regulator of splicing *(trs)* (217). Further studies have revealed, however, that the effect of Rev is primarily on the cytoplasmic mRNA and that the levels of spliced and unspliced viral RNA remained unchanged in the nucleus in the presence or

absence of Rev (301, 302, 311, 312). Thus came the proposal that Rev induces structural gene expression by activating the sequence-specific nuclear export of incompletely spliced and unspliced RNA species. The role of splicing in Rev action was less favored mainly due to the observation that the cytoplasmic expression of a nonspliceable HIV-1 *env* gene sequence is also subject to Rev regulation (301). A subsequent study, however, showed that recognition of splice sites in addition to the presence of RRE is a must for Rev responsiveness (313). This study also showed that rapid splicing due to the presence of efficient splice sites precluded Rev responsiveness. The retention of incompletely spliced HIV RNAs within the nucleus, in the absence of Rev, appears to be due to the formation of splicing complexes that are slow in executing splicing because of inefficient splice sites. Rev probably promotes dissociation of the RRE-containing unspliced RNAs from these complexes and makes them available for export. The earlier observation that a nonspliceable HIV-1 *env* gene sequence, containing only one splice site, was also subject to Rev regulation (301), could be explained by the fact that a lone splice site, in the absence of the other, can still be recognized by splicing components. Retroviruses in general appear to contain inefficient splicing sites, which may account for the accumulation of an unusually large amount of unspliced viral RNAs in the nucleus (314, 315). This accumulation may reflect a requirement for transporting unspliced RNA to the cytoplasm. Mutations that enhance the rate of viral RNA splicing have been shown to result in defective replication (314, 316). Alternatively, Rev may act independently of spliceosome formation and establish an alternative transport pathway for RRE-containing mRNAs.

The role of the CRS elements is not clear. They may act as nuclear retention signals by virtue of binding to some cellular factor(s), and Rev-RRE interaction may counteract this nuclear retention effect. In some experiments, HIV-1 mRNAs made from recombinant plasmids accumulate in both the nucleus and the cytoplasm of the transfected cells in the absence of Rev, yet no envelope protein is made from these mRNAs even though they are polysome-associated (312, 317, 318). Evidently, under some conditions, the CRS elements can specify a block to the translation of cytoplasmic mRNA, which is overcome by Rev. This is somewhat similar to the observation made about posttranscriptional effect of Tat (as discussed in section VA).

C. Transcriptional Down-Regulation by Rev

Rev appears to exhibit a dual regulatory role in determining the level of virus expression. At low levels, Rev stimulates virus expression by its posttranscriptional transactivation property, whereas at high level it appears to down-regulate viral transcription. A *rev*$^{-}$ mutant of HIV-1, though replication-defective, expressed elevated levels of the 1.8 kb mRNA species but little or

no higher-molecular-weight species (297). Further study has shown that Rev at relatively high levels exerts a negative effect on transcription in a dose-dependent manner (319). The *cis*-acting sequence responsive to Rev down-regulation appears to be located in the LTR upstream from −117. The inhibitory effect of Rev on transcription is not restricted only to HIV LTR; other promoters such as the immediate early promoter of cytomegalovirus (CMV), papovavirus JC promoter, and to a lesser extent the RSV LTR are also negatively affected. It is worthwhile to note that Rex, the HTLV protein functionally analogous to Rev, also down-regulates HTLV-LTR-directed gene expression when overexpressed (320). This observation strongly suggests an important role for this transcriptional inhibitory property of Rev in HIV pathogenesis.

D. Is Nef a Negative Regulator?

Whether Nef protein negatively regulates virus expression or not is a controversial issue. Using isogenic strains of HIV-1 that differ only in their ability to produce Nef protein, it was found that *nef*-defective viruses replicate slightly faster than the wild-type viruses in $CD4^+$ T-cell lines (321–323). It was also reported that Nef down-regulates HIV-1 LTR-directed expression of heterologous genes (324, 325). Sequences within the LTR required for Nef activity were reported to be located either within the NRE or between the NRE and the site of RNA initiation.

In conflict with these studies, others have reported that isogenic strains of HIV-1 differing only in *nef* gene do not differ substantially in their ability to replicate in $CD4^+$ T-cell lines (326). Furthermore, it has also been shown that Nef does not inhibit transcription from HIV-1 LTR in a variety of cell types (327).

E. Activation of HIV by Other Viruses

Although HIV is the etiological agent of AIDS, other cofactors have been implicated to play a role in the outcome of HIV infection, which ranges from asymptomatic carrier state to full-blown AIDS, as well as from latency to reactivation. Several viruses commonly detected in AIDS patients such as herpesviruses (328–330), papovaviruses (330), adenoviruses (331), HTLV-I (332), and human herpesvirus-6 (HHV-6) (333) are capable of transactivating the HIV-1 LTR. Many of these viruses infect and replicate in the same cell type as HIV-1. Interestingly, coinfection of the same cell by HIV and human cytomegalovirus (HCMV) has been detected in the brain tissue of AIDS patients (334). Some of these viruses also encode transactivator proteins, which have been shown to increase expression of the HIV LTR. These viruses include HTLV-I, Cytomegalovirus (328), Herpes Simplex Virus (329, 330), Epstein Barr Virus (335), HHV-6 (333), Adenovirus (336), Papovaviruses

(330), and Hepatitis B Virus (337). These viruses may contribute to AIDS progression by transcriptional activation or reactivation of HIV as well as through additional mechanisms (for a recent review, see Ref. 338). Of particular relevance as a cofactor may be HTLV, since these viruses follow the same routes of transmission as HIV (blood transfusion, sex) and target many of the same risk populations, e.g. intravenous drug users, hemophiliacs. In fact, epidemiological data indicate that patients doubly infected with HIV and HTLV have a much worse prognosis than those infected with HIV alone (339).

VI. IMPACT OF TAT ON CELLULAR FUNCTIONS

Like most nuclear transactivators, Tat was expected to restrict its action to the cell in which it was produced. However, recent studies suggest that Tat may also function as an exogenous factor, based on observations that indicate that Tat is found extracellularly, and is taken up by other cells in a biologically active form. Purified Tat protein placed in the culture medium is taken up by the cells and is able to transactivate HIV LTR-directed gene expression (293, 340).

The second exon of Tat, which is completely dispensable for transactivation, contains a highly conserved tripeptide sequence Arg-Gly-Glu (RGD), which is a characteristic of many extracellular proteins that bind to cell adhesion molecules (341). RGD-containing peptides were found to compete with full-length Tat protein for binding to the cell surface (342). Since the one-exon Tat protein lacking the RGD sequence is also taken up by cells (340), it appears that the interaction of Tat with the cell surface through the RGD sequence is not involved in internalization. This interaction, however, may serve some other function such as cell-cell interaction through adhesion. A cell surface receptor for Tat has recently been identified as a novel integrin composed of the vitronectin receptor α_v subunit (α_v) and a β subunit that has not been identified previously (B. Vogel, S.-J. Lee, F. Wong-Staal, W. Craig, M. Pierschbacher, and E. Ruoslahti, unpublished observation). Interestingly, this interaction primarily requires the basic domain of Tat, while the RGD sequence possibly plays a secondary role. Since the basic domain is encoded by the first exon, it is possible that the interaction of Tat with this novel integrin is responsible for internalization. However, the biological significance of this interaction remains to be established.

Extracellular Tat protein has been detected in the medium of HIV-1-infected cells as well as cells transfected with *tat* DNA (343). The extracellular Tat is likely derived by secretion from the cell, since there was no correlation of the amount of Tat found in the medium with extent of cell lysis. Although Tat does not contain a classical signal peptide, a number of cytokines, such as IL-1α, IL-1β, acidic and basic fibroblast growth factors, and

platelet-derived endothelial growth factors, are secreted from cells in spite of the lack of a known signal peptide. Therefore, these cytokines, as well as Tat, may follow a secretory pathway yet to be elucidated.

A novel effect of Tat is its ability to affect cell proliferation as an exogenous factor. Two systems have been described. First, Tat inhibits antigen-induced proliferation of lymphocytes when added to the culture medium (344). This effect is specific as oxidized and mutant Tat proteins are inactive, and the inhibitory effect is abrogated by antibodies against Tat. The amount of exogenous Tat protein required to observe such an effect is comparable to that required for transactivation of a reporter gene by extracellular Tat. It was proposed that this may be one pathway whereby HIV can exert an effect on the immune system. The physiological significance of this observation, however, remains unclear, as it is unlikely that circulating Tat level reaches sufficiently high to exert a general regulatory effect.

A second system relates to the stimulatory effect of extracellular Tat protein on the growth of cell lines derived from Kaposi's Sarcoma tissues (343), and suggests that Tat may play a contributory role in the progression of KS. KS is an angiosarcoma of mesenchymal/endothelial origin frequently occurring in elderly men of African and Mediterranean origin as well as in immunosuppressed individuals. The incidence of Kaposi's Sarcoma, however, is increased a thousandfold in certain populations infected with HIV-1. Although HIV infection is a predisposing factor for the development of KS, HIV sequences are not found in the tumor cells, suggesting an indirect role of the virus (345–347). Similarly, transgenic mice carrying the *tat* gene, which develop KS-like lesions, express *tat* in the dermal tissues but not in the tumor cells (348). Several cell lines have been established from Kaposi's lesions of AIDS patients (349). These cell lines share many characteristics of the original tumor cells. For example, the cells are spindle-shaped and induce neoangiogenesis as well as KS-like lesions in nude mice (350). They also release a variety of cytokines with autocrine and paracrine growth effects (351). These cell lines were initially established using conditioned media from T-cells infected with human retroviruses (HTLV-I, HTLV-II, and HIV-1). These same conditioned media continue to enhance proliferation of the KS-derived cell lines in culture. The growth-promoting activity present in HIV-1-infected cell-conditioned medium turned out to be Tat protein itself as this activity is blocked by antibodies against Tat (343). Moreover, recombinant Tat protein was also found to have the same growth-promoting properties. The growth-promoting effect of Tat was detected at very low concentrations, which may be attained under physiological conditions. The optimal threshold of Tat for growth-promoting activity is much lower than that required for LTR transactivation, suggesting that the two phenomena may occur by different mechanisms. It is not clear whether Tat released from infected cell is taken up by the target cells and activates cellular genes

required for growth promotion, or whether Tat acts directly as a growth factor and stimulates cell proliferation by signal transduction. Since Tat protein does not stimulate the growth of smooth muscle cells or endothelial cells, the putative progenitors of the KS cells, it is unlikely that Tat by itself induces Kaposi's Sarcoma. This idea is entirely consistent with epidemiological studies, which indicate that HIV-infection may increase the risk for KS, but is neither necessary nor sufficient for induction of the disease.

VII. GENETIC HETEROGENEITY

Human and related simian immunodeficiency viruses (SIVs) exhibit a remarkable degree of plasticity in their genomes. Most of the analyses on genomic diversity so far have been carried out with HIV-1, but similar diversity is apparent of HIV-2 and various SIVs. One can study the divergence of these viruses at several levels. First, one can follow the complexity of the virus population in a single individual in the course of infection. This type of study gives some interesting insights into the various factors that may be at play in the generation of genetic variants, as well as the implications of such variation on disease progression and attempts at viral prevention and intervention. On a broader scale, one can assess the extent of variation among viruses obtained from different individuals. This comparison may be useful as a molecular epidemiological approach to determine virus transmission among potential donors and recipients. More importantly, in conjunction with immunological determinations, it also helps to circumscribe the regions that are important for vaccine development. Finally, the nature and degree of homology between the related human and simian immunodeficiency viruses will shed some light on the evolution and origin of this virus family.

A. Genomic Complexity and Evolution in a Single Infection

The idea that each isolate of HIV-1 represents a single genetic entity was dispelled with the discovery of coexisting genotypes in in vitro cultured virus "strains" or uncultured tissue samples from infected individuals. Using restriction enzyme mapping of multiple virus clones from two patients, Saag et al (352) found that although in general clones obtained from the same patient are more related to each other, they are also distinguishable on the basis of their unique set of restriction enzyme sites. Goodenow et al (353) extended these studies, using the polymerase chain reaction (PCR) to amplify and sequence various regions of the HIV genome. They also compared such analyses on virus genomes that had been passaged in culture with those that were directly amplified from patient's peripheral blood mononuclear cells. Several conclusions can be drawn from these studies: (*a*) there is an inordinate degree of genetic complexity in HIV and that each "isolate" consists of a mixture or "swarm" of microvariants that are highly related but distinguish-

able. This finding is similar to that of other RNA viruses, giving rise to the concept of a "quasispecies" (354). (*b*) The predominant genotypes that are found in uncultured tissues are different from those present in cultured cells, suggesting that in vitro cultivation imposes a selective process (353). (*c*) There is a prevalence of defective genomes characterized by premature termination codons, insertions, deletions, and substitutions in several of the essential genes, which would prevent virus replication (353). Whether these defective genomes play a role in pathogenesis is an intriguing question.

Highly related HIV genomes obtained from the same patient can have very different biological properties with respect to replication efficiency, tropism, and susceptibility to virus neutralization (355, 356). For example, Koyanagi & Chen (355) found that virus isolates obtained from the brain and cerebral spinal fluid from the same patient could both replicate in peripheral blood lymphocytes, but only virus from the brain could efficiently infect macrophage/monocytes. Looney et al found that a single amino acid substitution in a critical region of the virus envelope could greatly affect the susceptibility of different cloned virus variants to neutralizing antibodies (356). These observations suggest that the genetic composition of an HIV quasispecies will also determine its predominant biological phenotype, and furthermore, such composition may change under selective pressures in the infected host. This concept of a rapidly evolving virus population also gains support from studies of sequential isolates. The coexistence of mixed genotypes makes it more difficult to assess divergence of the virus with time of infection. Thus, the earlier observation of differences in sequentially obtained isolates (357) could be due to selection from an existing population rather than change over time. However, in comparing the biological phenotypes of sequential isolates from patients, Asjo, Fenyo, and colleagues (358, 359) made the interesting observation that isolates obtained from asymptomatic patients in general display restricted growth in T-cell lines and reduced cytopathic effect (a slow/low phenotype), while viruses obtained from advanced AIDS patients infect T-cell lines readily, with a rapid rise in reverse transcriptase activity and production of large syncytia (a rapid/high phenotype). In some patients, a progression to the more highly cytopathic isolates occurs with progression to disease in the same host (360). In the one case study of a laboratory worker infected with an extensively tissue culture–passaged virus, HIV-IIIB, the emergence of variants that are resistant to neutralizing antisera raised against the parental virus was observed in the course of infection (W. Blattner, personal communication).

What may be some of the selective pressures for change? We hinted above at immunoselection as a force, similar to what has been observed in another lentivirus system, namely, equine infectious anemia virus (361). This possibility is also supported by in vitro studies, which demonstrated the generation of escape mutants when the virus is passaged in the presence of a

selecting neutralizing serum (362). Similarly, resistant mutants can also be selected for in the presence of an antiviral drug in vivo, such as azidothymidine (AZT) (363). The emergence of more "virulent" forms of the virus, however, may represent removal of selective pressures rather than positive selection. A fast-replicating, highly cytopathic virus may be rapidly cleared by the immunosurveillance of a healthy infected host early after infection, and therefore a slow/low virus is selected for. As this low-level virus expression erodes the immune system, a more replication-efficient virus is allowed to dominate, which in turn causes more rapid deterioration of the immune system. Thus, one can envision a vicious cycle of ever-increasing viral virulence, and ever-decreasing immune competence of the host. This scenario also explains why the virus does not become more virulent with time in the course of the epidemic.

Selective pressure is only one consideration in retrovirus evolution. Another is the accumulation of mutations, predominantly as a function of the reverse transcription process, which is more error prone than DNA replication owing to the lack of a proofreading mechanism (97). The extent of mutation is therefore dependent on the error frequency of the reverse transcriptase and the number of replication cycles in a given time. HIV-1 undergoes more active replication than certain other retroviruses such as HTLV-1. In addition, HIV RT is found to have unusually poor fidelity, with an estimated error rate of 1 substitution in every 1700 to 4000 polymerized nucleotides (189, 190), which is an order of magnitude higher than that of the avian myeloblastosis virus or murine leukemia virus RT. Furthermore, these studies revealed that certain target sequences yielded a significantly higher error frequency, suggesting that there may be "hotspots" for mutation on the virus genome. In addition to mutational events, recombination among existing genotypes can also contribute to the generation of diversity.

B. Divergence of HIV-1 in the Population

The degree of similarity among any two HIV isolates is in general less than that among viruses from the same person, and depends on the time they have diverged from a common progenitor. Therefore, it is expected that viruses derived from donor-recipient pairs would be highly related, and viruses from the same cohorts and same geographical areas more so than those from distant populations. Furthermore, the extent of divergence increases with the length of time the virus has been in a given population. For example, viruses from Africa are more divergent than viruses in the United States and Europe, supporting the notion that the virus originated in Africa. Attempts have been made to calculate the time of entry of HIV into man based on the genetic distance between isolates having a known time of divergence (364). Such calibrations, however, should be taken with caution since the necessary

assumptions for constant mutation rate and replication rate are likely to be invalid.

One major implication of the extensive heterogeneity of HIV is its impact on vaccine development. This concern was fueled by the findings that the most divergent part of the genome was in fact the envelope gene (365, 366), which encodes critical products for immune recognition, and that the viral envelope protein indeed elicited predominantly a type-specific neutralization response (367). Amino acid variability can be as great as 15% over gp160, the envelope precursor protein, with discrete regions of conserved and hypervariable regions (260). As expected, there are functional constraints on the envelope protein that limit its variability. For example, all of the 18 cysteine residues in gp120 are conserved, presumably to maintain the tertiary structure conferred by the disulfide bonds. The region in gp120 responsible for binding to the cellular receptor, CD4, and that in gp41 for inducing cell fusion are also highly conserved. One might speculate that the hypervariable regions have no specific function other than to maintain the proper conformation of the envelope protein, and therefore mutations are more tolerated. Of interest, one of the hypervariable regions (V3) is recognized by both neutralizing antibodies (368) and cytotoxic T-lymphocytes (369), and immunologic selection may well be the mechanism for generating diversity at this site. The fact that the highly variable V3 sequence (as much as 50% variability among HIV-1 isolates) also appears to be the major immunodominant neutralization epitope(s) makes it both an attractive and a formidable target for vaccine development. The V3 sequence contains a loop formed by a disulfide bridge between two invariant cysteines at positions 303 and 338 (370). At the crown of the loop is a relatively conserved tripeptide G-P-G, which presumably allows the protein to adopt a β bend structure. Recently, the V3 loop sequence was determined for a large number of HIV field isolates using PCR amplification and sequencing (370). Results from this study revealed that variation in this region is much less extensive than previously thought based on the few laboratory isolates. There is a greater than 80% conservation in 9 out of 14 amino acid positions in the central portion of the loop and a strong preference for particular oligopeptide sequences. Furthermore, the predicted structural motif (β strand–type II β turn–β strand–α-helix) of the consensus sequence is also prevalent. Of practical importance is the finding that antibodies that bind the consensus peptide sequence GPGRAF were able to neutralize 60% of random HIV isolates (371).

C. Evolutionary Divergence of the Human and Simian Immunodeficiency Viruses

Two related but distinct immunodeficiency associated human lentiviruses have now been identified: HIV-1, the prototype AIDS virus prevalent in the

United States, Central Africa, Europe, and throughout most of the world, and HIV-2, which is largely confined to regions of West Africa and seems to exhibit a lesser virulence or penetrance than HIV-1. In addition, nonhuman primate lentiviruses have been obtained from African green monkeys (SIVagm), sooty mangabey monkeys (SIVsmm), baboons (SIVmnd), and captive rhesus monkeys (SIVmac). Most recently, a related virus was isolated from chimpanzees (SIVcpz) (372). Of these primates, the African green monkeys, sooty mangabey monkeys, and mandrill baboons appear to be infected in nature, while the others are more likely to be infected in captivity. Several parameters indicate that SIV has been indigenous to the three naturally infected monkey populations for some time: (*a*) Infection is prevalent. For example, 20–50% of green monkeys in Kenya, Ethiopia, and South Africa have antibodies to SIV proteins (373). (*b*) Lack of disease induction in either naturally infected or experimentally inoculated monkeys, suggesting a virus-host adaptation through coevolution. In contrast, the macaque monkeys are highly susceptible to disease induction by SIV. (*c*) More extensive variability. Using divergence in the *pol* gene as a measure, it is apparent that SIV has been in African green monkeys and sooty mangabey monkeys much longer than HIV-1 or HIV-2 has been in man. Up to 20% variation has been observed among SIVs from these monkeys, while the HIVs only vary as much as 7% in each group.

The question then arises as to whether some of these "older" SIVs could have been a precursor to other SIVs and to the HIVs. Nucleotide sequence analysis revealed four distinct groupings of primate lentiviruses, each about 55–60% related in their *pol* gene: HIV-1, SIVagm, SIVsmm, and SIVmnd being the prototypes of each group (Ref. 373, for review). Interestingly, SIVmac and HIV-2 both fall into the same group as SIVsmm, suggesting that macaques and humans were infected recently by a virus from sooty mangabey directly, or one closely related to it (374). Along the same argument, it is likely that the precursor to HIV-1 may be derived from a monkey virus, although the existence of such a virus has not been demonstrated. The virus obtained from captive chimpanzee was highly related to HIV-1 (372), but since chimpanzees are not commonly infected, it is possible that the chimpanzee was infected by a human virus. The evolutionary implications of these findings have to await additional isolations and genetic analyses.

VIII. CURRENT AND FUTURE PROSPECTS FOR AN AIDS VACCINE AND ANTIVIRAL THERAPY

The optimism for an AIDS vaccine has waxed and waned in the years since HIV was first demonstrated to be the cause of AIDS. The extensive genetic heterogeneity of HIV, the early unsuccessful experiments in animals (chimpanzees and rhesus monkeys), and the lack of a clear correlation of antiviral

immunity and disease progression in infected people were all foreboding observations. Recent progress, however, has injected a new sense of optimism for the prospect of designing a protective, broadly cross-reactive vaccine. As alluded to in Section VIIB, variability in the dominant neutralization and cytotoxic T-lymphocytes (CTL) epitope in gp120 was overestimated because of the choice of prototypes for comparison. It seems possible to induce a broad neutralization response (60% of random isolates) with a single peptide sequence (371). Furthermore, there is preliminary indication that serotype classes may exist in the human population, making it possible to use a cocktail approach to broaden the reactivity. In addition, other neutralization and T-cell epitopes have been mapped, and some of these are relatively conserved. More encouraging is the surge of positive results in monkeys where the vaccinated animals were shown to be protected either from infection or rapid disease induction by low-dose challenge viruses (375). The most effective immunogen in these studies seems to be whole-inactivated virus, but in one study, boosting with a V3-derived peptide in chimpanzees that were primed with inactivated HIV apparently played a key role in enhancing immunity (376), and in another study, recombinant gp160 conferred protection to two vaccinated chimpanzees (377). In view of these data, an AIDS vaccine for man is almost tangible. The difficult parts would be to assess safety and efficacy of candidate vaccines in man and to identify appropriate groups for clinical trials (378).

In contrast, the administration of antiviral drugs to AIDS patients would be less problematic, especially for those more advanced in disease. Nevertheless, to determine efficacy of a drug, the proper clinical trial with placebo controls is still mandatory. So far, the best known antiviral drugs for HIV are the family of 2'-3' dideoxynucleoside analogs, most notably 3'-azido-2',3'-dideoxythymidine (AZT) which has been proven for clinical use and has proven efficacy in prolonging patients' life span (379). Other compounds in this family, including 2',3'-dideoxycytidine (ddC) and 2',3'-dideoxyinosine (ddI) are also undergoing phase I/II clinical trials. Although these compounds have demonstrated antiviral activity in vitro and in vivo, there are also mitigating factors for their long-term use at high doses, such as toxicity of the drugs and emergence of drug-resistant mutants. For example, administration of AZT was found to be associated with bone marrow suppression, which warranted transfusions or dose reduction in one third of patients during the first six months of trial (380, 381). Furthermore, AZT-resistant mutants were isolated from patients who had been under therapy for more than six months (363). Such mutants are not cross-resistant to the other dideoxynucleoside analogs. It is hoped that using a combination regimen of more than one drug, one can lower the dosage of each drug, and thereby lower the toxicity and propensity for developing drug resistance.

The dideoxynucleoside analogs inhibit the reverse transcription process by acting as a chain terminator when incorporated into the viral DNA. There are many other steps in the HIV replication cycle that are amenable to interruption. Like the reverse transcriptase, the other retroviral enzymes protease and integrase are also potentially crucial targets. Synthetic peptides representing transition-state analogs of the protease substrate have been shown to be effective in inhibiting viral protease function as well as HIV replication in vitro, with little effect against cellular aspartic proteases (382–384). These results portend exciting new approaches for treatment of HIV infection.

In addition to targets that are common to all retroviruses, HIV also offers unique opportunities for intervention. Since the first identification of the CD4 molecule as a critical component of cellular receptor for HIV, different lines of approach have been taken to exploit this observation to the end of therapy. It was first shown that soluble recombinant CD4 (rCD4) acted as a competitor for HIV binding and inhibited virus replication in target T-cells (47–49, 80, 385). Attempts were also made to generate chimeric CD4-Ig molecules that make up the amino-terminal portion of CD4, which contains the gp120 binding site and the constant heavy chain domain of human (386) or murine (387) immunoglobulins. These "immunoadhesin" molecules retain antiviral activity and in some instances also mediate antibody-dependent cell cytotoxicity (ADCC). Furthcr, they have the advantages of a longcr plasma half-life, capacity to cross the placenta as well as to bind Fc receptors with high affinity. There is yet no evidence of clinical efficacy of either rCD4 or the immunoadhesin. At the preclinical level, soluble CD4 or monoclonal antibodies to gp120 have also been used as targeting devices that deliver toxin molecules to the infected cells to selectively destroy that cell population (388, 389).

A number of molecular approaches have been examined for their potential usefulness in virus inhibition. The following discussion is not meant to be exhaustive, but to indicate the trends of research in this area. As pointed out earlier, HIV replication is mediated by two essential transactivation pathways, both involving an RNA-binding protein (Tat and Rev) recognizing an RNA target sequence (TAR and RRE, respectively). In addition, cellular proteins participate in these pathways by interacting with the viral protein or RNA target. TAR-RNA decoys (390; J. Rappaport, personal communication) and antisense oligonucleotides directed at RRE (S. Daefler, M. Klotman, and F. Wong-Staal, unpublished), which would interfere with the natural RNA target binding to viral proteins, have been shown to inhibit transactivation and virus replication. Similarly, *rev* mutants with a negative dominant phenotype (223), which can still bind to RRE but may be unable to react with a cellular factor, can inhibit wild-type Rev function. Antisense oligonucleotides di-

rected at the *tat/rev* coding sequences have also been shown to suppress virus expression greatly (391).

A somewhat novel technique to deplete viral-specific mRNA selectively is through the use of "ribozymes," catalytic RNA molecules with sequence-specific cleavage activity (392). Human cells expressing a hammerhead ribozyme targeted against the HIV *gag* gene transcripts were relatively resistant to HIV infection (393). The successful transition of these various genetic approaches to clinical application will depend critically on the technological advances in the frontier of gene therapy, which itself is a rapidly developing area.

In conclusion, there is a justifiable optimism for the prospects of successful treatment and prevention of HIV infection through the development of drugs and vaccine. The realization of these potentials may be too late, however, for those already inflicted with the disease and for those currently at risk of infection. In the meantime, public education and awareness are still the most powerful means to limit the spread of HIV and AIDS.

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