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Genetic Regulation of Human Immunodeficiency Virus

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INTRODUCTION

Although the discovery of the etiological agent of AIDS (human immunodeficiency virus [HIV]) is relatively recent, the wealth of information associated with this virus has far surpassed that for any other known human retrovirus. Yet, despite this growing body of knowledge, many aspects about the life cycle of HIV remain to be elucidated. The regulation of HIV gene expression is a tightly controlled process in which cellular and viral factors participate to allow the differential and temporal expression of viral gene products, a number of which are not found in most other animal retroviruses. In particular, two HIV regulatory genes, *tat* and *rev*, play key roles in the HIV life cycle and may provide attractive targets for antiviral therapy. Furthermore, their mechanisms of action are entirely novel and may be important paradigms of eukaryotic gene regulation.

REPLICATION OF HIV

The life cycle of HIV is similar to that of other known animal retroviruses. The progressive immunodeficiency of the disease is characterized by depletion of $CD4^+$ helper/inducer lymphocytes, at least partially resulting from the tropism of HIV for the $CD4^+$ subset of T lymphocytes and its cytopathic effect (61). HIV tropism appears to be restricted at the stage of viral entry since transfection of human and animal fibroblasts with viral DNA led to the generation of infectious virus (26, 63). Upon binding and adsorption into the target cell, HIV initiates the processes of uncoating, reverse transcription, and integration. The newly integrated proviral DNA thus persists and becomes part of the genetic endowment of the host cell. The propensity for HIV to establish a latent infection within the host is a hallmark of all the human T-lymphotropic retroviruses, which could remain hidden inside the very cells which are responsible for cell-mediated immunity against virus infection. The remaining steps of the replicative cycle include the synthesis of viral RNA, translation, assembly of mature virions, and egress from the infected cell.

Infection begins with the binding of the viral envelope to the cellular receptor CD4, found primarily on T-helper lymphocytes and to a lesser extent on monocytes, macrophages, follicular dendritic cells, and microglial cells. Normally the receptor for class II major histocompatibility complex molecules on antigen-presenting cells, CD4 binds the major viral envelope protein gp120 with an extremely high affinity. Once HIV binds to the target cell, entry into the cytoplasm occurs by a process independent of receptor-mediated endocytosis or pH-dependent entry via endosomes (67, 110). Most likely, a fusion event between the virus envelope and cell membrane occurs and the virus particle gains entry into the cell to initiate the replication cycle. It is also likely that an additional host factor is associated with this fusion event, since it was observed that murine cells

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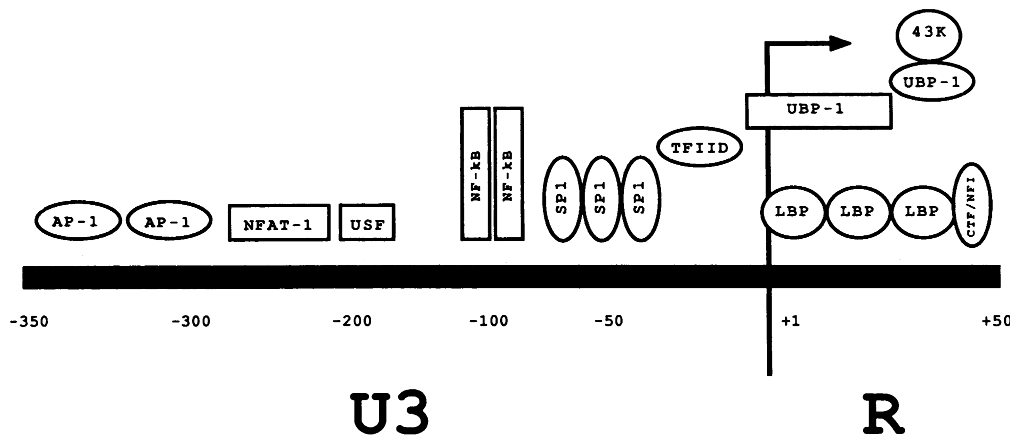

FIG. 1. *cis*-Acting elements found in the HIV LTR. Numbers represent distance from the start of transcription.

expressing human CD4 permitted viral binding but not infection (66). Hence, a cellular component(s) may be required to associate directly or indirectly with the CD4 receptor for productive infection by HIV.

After entry, uncoating of the virion takes place and the single-stranded genomic RNA molecule, still associated with core proteins, is converted into double-stranded DNA by the viral reverse transcriptase. The newly synthesized DNA (provirus) migrates to the nucleus, where the process of integration into the host genome occurs via the virally encoded integrase enzyme. Once the proviral DNA has integrated into the host genome, it remains there indefinitely and can be completely silent, as in a quiescent state of infection, or be actively expressed into progeny virus. In contrast, infection of resting T cells by HIV results in the initiation of viral DNA synthesis; however, the conversion of genomic RNA to double-stranded proviral DNA is incomplete, suggesting that additional factors necessary for the complete reverse transcription of proviral DNA are present in the activated T cell (122). The partial provirus is unstable and has a half-life of about 14 days.

Like other retroviruses, replication of HIV is determined at least in part by the state of proliferation and activation of the infected cells. Upon stimulation of the latently infected cells, synthesis of RNA takes place; these RNA molecules serve both as templates for viral protein synthesis and as RNA genomes to be packaged into progeny virions. Assembly of the immature virion occurs at the inner surface of the cell membrane, where the structural core proteins have aggregated. The association of two viral genomic RNA molecules with the smaller core proteins also occurs, and the nucleoprotein complex is packaged into the particle at the cell surface. Simultaneous with the assembly of the capsid precursor is the accumulation of viral envelope proteins on the outer surface of the cell. Once assembly is complete, the virus is released from the cell surface, where the envelope proteins undergo proteolytic cleavage by the virally encoded protease enzyme, forming the mature HIV virion.

LTR AND CELLULAR TRANSCRIPTION FACTORS

As for other retroviruses, the DNA regulatory sequences found within the HIV long terminal repeat (LTR) interact with a variety of cellular transcription factors which in turn may regulate viral gene expression from the HIV promoter. Mutations and deletions of these specific regions affect the rate of LTR-directed transcription and DNA binding of host transcription factors. The LTR, composed of the U3, R, and U5 regions, has been shown to be active in many different cell types. The sequence elements important for the promoter function of the LTR have been partially characterized (Fig. 1). The TATA box homology located at position −22 (relative to the start of mRNA transcription) is an essential element for *trans*-activation, functioning to correctly initiate the start of transcription by serving as the binding site for the TATA box DNA-binding protein TFIID. Immediately upstream from the TATA box are three G+C-rich sequences (−46 to −78) which bind the cellular transcription factor SP1 (38). Point mutations within the SP1-binding sites between −46 and −66 greatly reduced the rate of transcription both in vitro and in vivo (57), suggesting that viral transcription may be partially dependent on the interactions of SP1 with the HIV LTR. The enhancer element located between positions −109 and −79 contains two imperfect repeat sequences which have been shown to stimulate the rate of RNA transcription initiation from the LTR (89). Contained within the enhancer is the consensus recognition sequence for the DNA-binding protein NFκB (76), the activated T-cell protein HIVEN86A (34), and an additional enhancer-binding protein, termed EBP-1 (121). NFκB, an inducible transcription factor expressed in immunoglobulin-producing B cells, has also been found to be expressed in activated T cells and macrophages. This protein may impart a novel activation signal by which HIV-infected T cells, upon mitogenic stimulation, begin virus production. The interaction between NFκB and the HIV enhancerlike sequence may activate HIV gene expression by binding and initiating viral transcription from the LTR by a mechanism not unlike that proposed for the transcriptional activation of interleukin-2 (IL-2) and the IL-2 receptor α subunit (6). Thus, the potential interaction of NFkB and the enhancer-binding site may provide a clue to the activation of virus production seen in infected T cells in vivo.

Additional DNA-binding proteins have been identified which bind to the upstream LTR region. The nuclear factor of activated T cells (NFAT-1), AP-1, and upstream binding factor bind to the 5′ region of the LTR which also contains the negative regulatory element (35, 38, 103). The NRE, located at positions −420 and −159, functions to down-regulate the rate of transcription. Deletions within this region have led to higher virus production in $CD4^+$ T lymphocytes (64), suggesting that certain cellular proteins

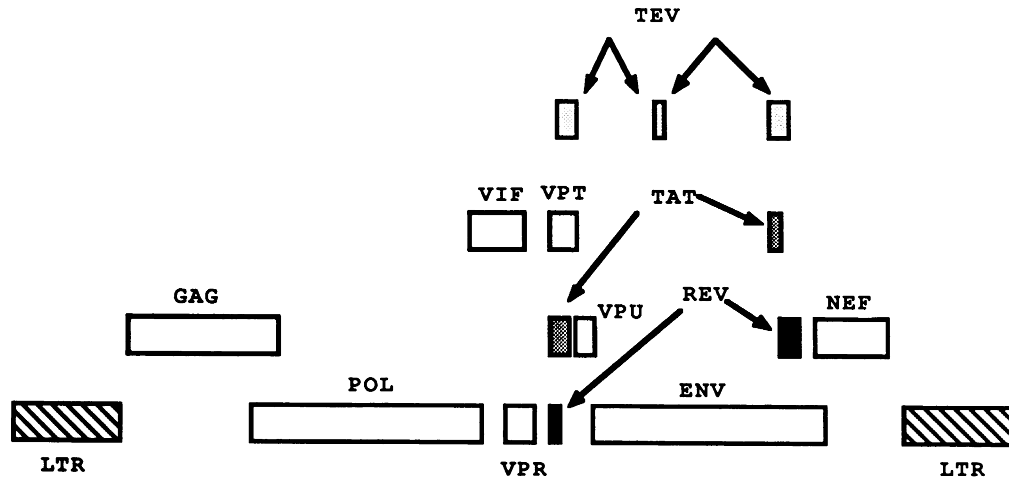

FIG. 2. Genomic structure of HIV-1. The nomenclature of each gene is described in Table 1.

bound to this region may have an inhibitory function on viral transcription. Three DNA-binding proteins which bind to the 5′ untranslated leader region have also been identified. CTF/NF-1 and leader-binding protein (LBP-1) are cellular transcription factors which interact with sequences immediately downstream from the mRNA start site to enhance transcription (58). LBP-1 has three distinct binding sites at positions −3 to +2, +8 to +12, and +28 to +32, suggesting a possible role in correctly initiating RNA transcription from the LTR. The *trans*-activation response sequence (TAR)-binding protein UBP-1 binds directly to the TATA box homology through the start of transcription to +44 and may function in the transcriptional activation of HIV gene expression (121). The complement of cellular factors that interact with the HIV LTR and regulate its expression is far from being fully delineated. It is expected that the variety and abundance of these factors will vary according to cell types (e.g., T lymphocytes versus monocytes) and cell cycle. The interplay of these cellular factors and viral *trans*-acting factors will determine the level of virus expression and ultimately the clinical course of infection.

MOLECULAR STRUCTURE OF THE HIV GENOME

Although expression of the simpler type C retroviruses is regulated predominantly by cellular factors impacting on the viral LTR, the human retroviruses and their animal counterparts also encode *trans*-acting viral regulatory factors. The organization of the HIV genome is now well defined (Fig. 2). HIV contains the three essential genes common to all retroviruses: *gag*, *pol*, and *env* flanked by the LTR promoter and enhancer regions. The *gag* gene encodes the virion proteins which form the inner matrix of the mature virus particle. The Gag polyprotein precursor p55 is cleaved during virus maturation, yielding several smaller proteins which include p17 (myristylated Gag protein), p24 (major core antigen), p7 (nucleoprotein), and p9 (nucleocapsid protein). The *pol* gene is expressed from a *gag-pol* polyprotein precursor, formed by a ribosomal frameshifting event mediated by a sequence located in the *gag-pol* overlap. A −1 shift in the reading frame allows the ribosomes to continue translation through the UAG termination site on the *gag* reading frame (55, 118). The subsequent cleavage of the *gag-pol* precursor gives rise to three enzymes, protease, reverse transcriptase, and integrase, which are all necessary for viral replication. The *env* gene encodes the envelope proteins found on the surface of the virus particle which are necessary for binding and entry into the host cell. The Env precursor protein gp160 is heavily glycosylated and is processed through a critical endoproteolytic cleavage, forming the major envelope glycoprotein gp120 and the small transmembrane envelope protein gp41 (73).

Along with the structural genes of HIV are at least six additional accessory genes whose functions impart both positive and negative effects on the HIV life cycle. The gene products of *tat*, *rev*, *nef*, and *vpr* potentially contribute to regulation of viral gene expression in both a positive and a negative manner. Tat and Rev are novel in their transactivating functions, using *cis*-acting sequences found in RNA, rather than DNA, as their targets for *trans*-activation. Tat, which has been shown to be absolutely essential for virus replication, acts as a central switch which initiates the expression of all viral genes. However, the actual mechanism of *trans*-activation remains controversial and will be discussed in detail later. Rev, also an essential viral protein, functions to control the differential expression of both spliced and unspliced viral mRNAs, thereby allowing the accumulation and nuclear export of mRNAs encoding the structural genes necessary for virus assembly. Nef was originally thought of as a negative regulator of expression; however, its actual function during viral replication has come under intense scrutiny recently. Previous reports have shown that mutations in *nef* resulted in an approximate 10-fold increase in viral replication, suggesting that Nef may lower all viral gene expression by acting as a silencer. In contrast, other investigators have shown that although Nef is dispensable for virus replication, mutations within the gene do not give rise to an increase in viral replication. There are preliminary reports that Nef may function as a necessary factor for virus growth in primary monocytes and macrophages, providing the tropism for productive infection in these particular cell types (46a) and that a functional *nef* gene is highly selected for in monkeys infected with the simian immunodeficiency virus (SIV) (21a).

The *vpr* gene increases the rate of HIV infection and may function in *trans* by stimulating gene expression from the

TABLE 1. Role of the HIV accessory genes for virus replication

Gene	*trans*-Acting product	Function
tat	*trans*-Activator	High-level expression of all viral genes
rev	Regulation of virion protein expression	Accumulation of full-length and envelope mRNAs in the cytoplasm
nef	Negative factor	Role is currently undefined; proposed to be a negative regulatory factor on HIV gene expression
vpr	Viral protein R	*trans*-Acting protein present in virus particle
vpu	Viral protein U	Cytoplasmic protein which facilitates assembly and export
vif	Virion infectivity factor	Increases infectivity of virus particles
vpt	Viral protein T	Tat-T open reading frame fusion protein of unknown function
tev	*tat-env-rev* fusion protein	Hybrid protein of three exons, has *tat* and reduced *rev* activity

HIV LTR. However, its effect is quite modest (two- to threefold) and nonspecific (works on a broad spectrum of viral and cellular promoters) and the mode of action remains unclear (15). A recent study of the *vpr* gene product of HIV-2 suggested that this gene, although dispensable for viral replication in human T lymphocytes, may be essential for the productive infection of human macrophages. HIV-2 viruses containing a mutant *vpr* gene failed to replicate in macrophages (47).

In addition to the regulatory genes, there are accessory genes unique to the HIV-SIV family of viruses: *vif*, *vpu* (found in HIV-1 but not HIV-2), and *vpx* (found in HIV-2 but not HIV-1), the functions of which remain poorly defined. The Vif protein apparently plays a role in virus infectivity. Mutations in *vif* generated wild-type virus with a reduced efficiency to infect $CD4^+$ T lymphocytes (30, 111). However, the mechanism by which Vif exerts its function is not clear. Since Vif is not a virion-associated protein, it must affect late events in the life replication cycle. The *vpu* gene facilitates the release of mature virus particles. Cells infected by Vpu mutant viruses retain a higher level of intracellular viral proteins and express a lower level of extracellular virus (112, 114). The gene encoding Vpx is found exclusively on the HIV-2 and SIV genomes. Although the function of this protein is unknown, it may play a role in cell-specific tropism for HIV-2. Viruses containing mutations within the HIV-2 *vpx* gene failed to infect primary blood lymphocytes (43).

Two additional novel open reading frames have been identified. The *vpt* open reading frame of HIV-1, originally defined as T, overlaps coding sequences of *tat*, *rev*, and *vpu* (14) and is expressed as a Tat-T fusion protein through a ribosomal frameshift. The consensus "slippage" sequence is highly conserved among HIV-1 isolates, and the protein is made in in vitro translation systems. However, there is no evidence that this protein is expressed in vivo. Also unique to HIV-1 is the *tev* reading frame generated from a spliced mRNA which encodes a 28-kDa protein consisting of coding sequences from *tat*, *env*, and *rev* (2, 98). The chimeric protein is bifunctional and exhibits both Tat and Rev activities. Table 1 summarizes some of the properties of these genes and their products.

MECHANISMS OF Tat *trans*-ACTIVATION

Functional Domains of Tat

The first indication that HIV-1 encoded *trans*-acting regulatory genes was the demonstration that HIV-infected cells are capable of stimulating LTR-directed gene expression (108). This activity was later mapped to the *tat* gene (1, 107). Tat is a powerful *trans*-activator of viral gene expression and is essential for virus growth (20, 31, 94). The *tat* gene codes for an 86-amino-acid protein which is localized in the nucleus and concentrated predominantly in the nucleolus of the infected cell (51, 93). The *tat* mRNA is generated from multiple splicing events and is composed of at least three species. The most common form is a tripartite mRNA consisting of a first exon derived from the 5′ untranslated sequence, a coding exon for the first 72 amino acids of the protein, and a second coding exon for the last 14 amino acids (1). Additional species may contain upstream noncoding exons derived from the *pol* gene. Deletion analysis has revealed that the amino-terminal 58 amino acids in the first coding exon of *tat* is sufficient for *trans*-activation, albeit with reduced activity (104). The 58 amino acids of Tat contain three essential functional domains. The acidic amino-terminal domain of Tat consists of two glutamate residues and one aspartate residue at positions 2, 5, and 8 which are critical for *trans*-activation function (86). The location of these acidic residues is consistent with the formation of an amphipathic α-helix, which may be important for efficient *trans*-activation (42). Exchanging the entire domain with heterologous sequences which have the potential to form an acidic, amphipathic α-helix partially restored activity. A stretch of seven cysteine residues makes up the second domain. It is believed that this region is necessary for zinc ion-mediated dimerization of the Tat protein (32). Missense mutations within this cysteine-rich region have shown that all residues but one are critical for activity (37, 50, 93, 95). The carboxy-terminal domain consists of a stretch of basic amino acids (residues 49 to 57) necessary for nuclear localization (50, 93). Point mutations within this cluster of positively charged amino acids resulted in the accumulation of nonfunctional Tat protein in the cytoplasm. When placed within heterologous cytoplasmic proteins, the nuclear localization signal, GRKKR, can impart properties of nuclear localization to these proteins (93). Recently it was shown that a peptide containing the basic domain of Tat bound specifically to its RNA target, TAR (for *trans*-activation response sequence) in vitro, suggesting that this domain may serve not only to localize the protein to the nucleolus but also to interact specifically with TAR (91).

Tat and TAR Interactions

trans-activation by Tat requires a specific recognition of this effector protein with a target sequence, TAR, contained in the R region of the viral LTR (89). Thus, unlike many of the enhancer sequences and transcription factor-binding sites which are located upstream from the start of transcription, the TAR sequence is located in a region of the LTR which is transcribed. Extensive mutagenesis of this region has found that the sequences located between +1 and +42 with respect to the initiation site of viral transcription are those necessary for Tat responsiveness (49, 56, 75). The position and orientation of the TAR element is also critical for its function; inversion of the sequence or an increased spatial arrangement between TAR and the start of transcription diminishes Tat *trans*-activation activity (56, 75, 100).

The RNA encoded by TAR between +1 and +111 has the potential to form one or more stem-loop secondary structures which would be found in all newly transcribed viral mRNAs (75). Feng and Holland (29) have described experiments using nucleotide substitutions and shown that Tat *trans*-activation requires the presence of the sequence CUGGG (positions +30 to +34) within the first TAR RNA stem-loop structure. A more extensive analysis of the TAR hairpin structure has revealed a three-nucleotide bulge at the base of the hairpin which also seems critical, as mutations within this bulge dramatically reduce Tat *trans*-activation (4, 92). In contrast, mutations that altered the sequence but not the structure of the stem-loop had no effect on Tat responsiveness, suggesting that TAR may function as RNA, rather than DNA. This was further substantiated by Berkhout et al. (5), who showed that directing changes outside the TAR sequence which would prevent formation of the stem-loop in newly transcribed TAR RNA also effectively abolished *tat* responsiveness. Therefore, Tat must recognize the correct secondary stem-loop structure in the nascent RNA to execute its function.

Recently, purified Tat has been shown to interact directly with TAR RNA, but not TAR DNA (23, 91). Additionally, a single uridine nucleotide change within a three-nucleotide bulge structure (positions +23 to +25) of the TAR RNA stem-loop was sufficient to abolish both Tat binding and *trans*-activation (91). However, it appears that Tat responsiveness may not depend on the ability of the protein to bind the TAR RNA sequence alone. Deletions within the TAR RNA structure which have been shown to abolish Tat activity in vivo (92) did not disrupt Tat binding to this sequence in vitro. Therefore, additional regulatory factors must be required for *trans*-activation. Several groups have shown that cellular proteins recognize and bind TAR RNA. Two distinct host proteins isolated from HeLa cell nuclei bind specifically to TAR RNA as a complex (39, 40). Also, a 68-kDa protein purified from HeLa cell nuclei has shown RNA-binding specificity to TAR (71). Host factors which bind purified Tat in a protein-protein complex have also been identified. Two such proteins have been obtained from a eukaryotic protein expression library (78). Lastly, human chromosomes encoding host factors which may play a role in Tat *trans*-activation have been identified by using rodent-human hybrid cells (46, 81). The effects of Tat *trans*-activation on the HIV-1 LTR, normally reduced in rodent cell lines, was greatly increased by the addition of human chromosome 12 into the hybrid cells, further emphasizing the importance of host cellular factors which may contribute to the Tat *trans*-activation response.

Transcriptional and Posttranscriptional Activation by Tat

Although many aspects of the Tat protein have been studied in detail, the mechanism of Tat *trans*-activation remains unclear. All researchers agree that Tat activates viral gene expression from the LTR and thus acts as the central control switch for HIV replication. Initial studies suggested that Tat dramatically stimulates LTR-directed protein expression with only a slight increase in steady-state mRNA levels (26, 88). However, some studies also suggested that transcriptional activation alone can account for the observed stimulation of protein expression (51, 56, 62, 75, 85, 87, 120). The current consensus is that Tat activates at both transcriptional and posttranscriptional levels (16) and that the contribution of each component may depend on the nature of the target cell. The location of TAR near the start of transcription initiation in the DNA and the 5′ cap site in the viral mRNA makes such a bimodal mechanism feasible.

At the level of transcription, it has been proposed that Tat acts primarily as an antiterminator of RNA elongation (59). In the absence of Tat, viral transcripts were prematurely terminated approximately 60 nucleotides from the start of mRNA transcription. In the presence of Tat, transcription occurred through the termination site, suggesting that Tat may function to relieve a block to transcriptional elongation at this site. A subsequent study showed that Tat both increases the frequency of initiation and stabilizes transcriptional elongation from the TAR element on the LTR (62). To resolve the apparent paradox of an RNA-binding protein's ability to increase transcriptional initiation, it was proposed that Tat may activate LTR-directed transcription as part of a feedback regulatory mechanism in which Tat complexes with nascent TAR-containing mRNA and recruits transcription factors to the promoter region on the LTR to increase the rate of subsequent rounds of transcriptional initiation (102) (Fig. 3).

Several groups have used chimeric Tat proteins to define the role of Tat-TAR interaction in *trans*-activation. The results suggest that TAR may serve as an enhancer element to direct Tat to a region 5′ from the mRNA start site, where it can interact with promoter elements to increase transcription from the LTR. Berkhout et al. (3) constructed Tat-Jun hybrid proteins which were able to activate an HIV LTR containing four AP-1 binding sites inserted into the TAR element. In addition, they found that the efficiency of *trans*-activation was dependent on the presence of NFkB/SP1 sequences; deletions within these sites resulted in poor *trans*-activation of the viral LTR. However, the level of activation they observed is quite low, a fraction of the activity of the normal Tat activity. By constructing a chimeric protein consisting of Tat and the RNA-binding coat protein of bacteriophage MS2, Selby and Peterlin (101) showed that *trans*-activation can occur with HIV promoters containing either TAR or phage operator sequences. Finally, Southgate et al. (109) have constructed a hybrid protein consisting of Tat and the RNA-binding domain of another HIV-1 *trans*-activator, Rev. This fusion protein could *trans*-activate an HIV-1 LTR which has the TAR element replaced by the HIV *rev*-responsive element (RRE). Thus, the ability of the Tat-hybrid proteins to *trans*-activate the HIV LTR in the absence of TAR suggests that Tat alone, when directed to the proper site near the promoter, may activate viral gene expression without the requirement of TAR RNA and DNA-binding proteins. The interaction between Tat and TAR RNA may then serve to increase transcription from the LTR promoter region by a mechanism yet to be defined. However, these results do not explain why some TAR mutants which lack *trans*-activation activity could nonetheless bind Tat protein with equal avidity as the wild-type TAR does.

Although it appears that Tat functions primarily at the transcriptional level, there is evidence to suggest that it may also possess posttranscriptional activity. When TAR-containing chloramphenicol acetyltransferase (CAT) RNA was injected along with purified Tat into the nucleus of *Xenopus* oocytes, expression of the CAT reporter gene occurred even in the presence of transcriptional inhibitors (8). However, injection of purified Tat and CAT RNA into the cytoplasm resulted in the absence of any CAT gene expression. It was proposed that Tat may confer modifications of the TAR-containing mRNAs in the nucleus, which would increase their stability or eventual translatability. Furthermore, the same investigators (9) extended their studies to show that the

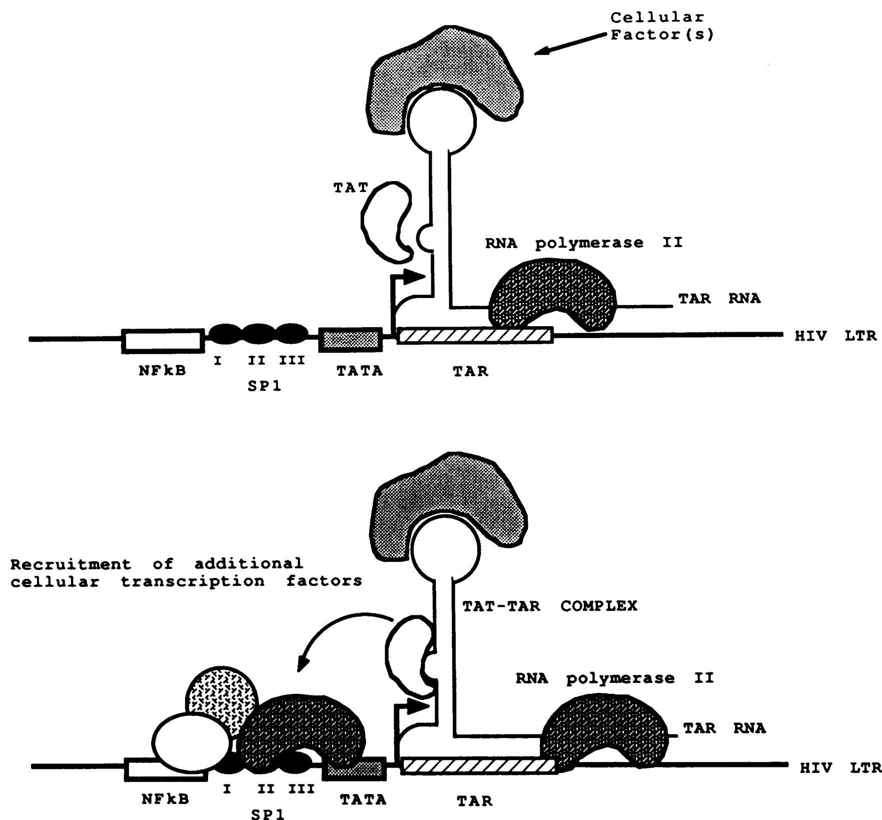

FIG. 3. Proposed mechanism of feedback regulation on HIV-1 transcription as a result of Tat-TAR interaction. The interaction shown here between Tat and TAR may facilitate the recruitment of additional cellular factors necessary to initiate transcription from the HIV LTR upstream U3 region.

stem-loop sequence within TAR is critical for nuclear activation of TAR RNA expression since TAR^- RNA synthesized from the HIV LTR was not translated in the cytoplasm of *Xenopus* oocytes. They postulate that the U3 region of the LTR may block the translation of all viral mRNAs and that the Tat-TAR interaction may function to remove a nuclear translational block imposed by the U3 promoter region of HIV-1. However, it is not clear whether these observations are relevant in a mammalian cell system. Taken together, these findings indicate that Tat protein, either alone or associated with cellular factor(s), may bind to the TAR RNA in the nucleus as part of a nucleoprotein complex to increase the probability of transcriptional chain elongation or reinitiation, as well as to enhance the translational efficiency or stability of the TAR-containing mRNA.

TEMPORAL REGULATION OF HIV GENE EXPRESSION BY Rev

One unique feature common to the human retroviruses is their ability to differentially regulate the expression of structural and regulatory gene products in a temporal manner. In a single cycle of infection (60) or virus replication in transfected cells (119), the early transcripts that appear are those that encode regulatory proteins (Tat, Rev, Tev, and Nef), while the late transcripts comprise those which code for proteins found predominantly in the mature virus particles (Gag, Pol, Env, and Vpr). The early-to-late switching is brought about by the Rev protein of HIV and the Rex protein of human T-lymphotropic virus (HTLV). Rev and Rex are novel *trans*-activator proteins which control the differential expression of viral proteins exclusively at the posttranscriptional level, by allowing the accumulation and utilization in the cytoplasm of unspliced and singly spliced viral mRNA (26, 54, 69, 106). In the absence of Rev or Rex, the only viral RNA species which accumulate in the cytoplasm are multiply spliced and encode only a subset of the viral regulatory proteins.

Rev Protein

The *rev* gene was originally discovered within the two *tat* coding exons after mutational analyses revealed that some deletions either within or near the *tat* sequences could not be complemented by functional *tat* cDNA clones (26, 106). The *rev* coding sequence is composed of several mRNA species that encode a protein consisting of 116 amino acids (26, 94). Rev is a 19-kDa phosphoprotein located predominantly in the nucleolus of the cell (13, 17, 27). At least two serine residues have been shown to be phosphorylated in vivo (48); however, phosphorylation of Rev appears to be nonessential for activity (68). Two distinct motifs critical for function have been identified on the Rev protein. A short basic stretch of amino acids at the amino terminus (residues 40 to 45) has been shown to be important for nuclear, and specifically nucleolar, localization (13, 68). Some mutations within the basic sequence resulted in the accumulation of inactive Rev protein in the nucleus, but not in the nucleolus (13, 115).

Thus, nucleolar localization of Rev is apparently required for full activity. The highly basic stretch of arginine residues has also been postulated to function as the sequence-specific RNA-binding domain which interacts with the *cis*-acting RRE. Recently, Olsen et al. (82) demonstrated that mutations within this basic stretch disrupted the ability of Rev to bind to the RRE sequence. A second region critical for Rev function is a leucine-rich sequence (residues 75 to 84) at the carboxy terminus. Mutational analysis suggests that this region may constitute an activation domain. Substitutions of the leucine residues resulted in the production of mutants with a transdominant negative phenotype, which effectively inhibited wild-type Rev function in cotransfection experiments (68).

Rev and RRE Interactions

The function of Rev is dependent on the presence of a *cis*-acting sequence (RRE) found within the *env* gene of HIV (69, 90). The location of the RRE within the *gag-pol* and *env* mRNAs further supports the working hypothesis that Rev functions as a posttranscriptional regulator of virus expression. RRE has been functionally defined to be a sequence of about 200 nucleotides (21, 83). This sequence on the RNA is predicted to form a complex secondary structure composed of five stem-loops (70, 83). Within this structure the most important determinant for Rev responsiveness was shown to be stem-loop II (70). Stem-loop II resembles a hammerhead-like configuration which can be subdivided into stem-loops IIA and IIB. Deletion mapping studies of the RRE secondary structure has revealed that deletions within stem-loop II or IIA abolish RRE activity while stem-loops III, IV, and V were dispensable for function (70). Deletions within stem-loop I reduced but did not abolish activity. The importance of these stem-loop structures is also consistent with the observation that the integrity of the secondary structure, rather than the primary nucleotide sequence, is the determining factor for Rev-RRE interactions (22, 83).

Purified Rev specifically binds to the RRE RNA in vitro (12, 18, 19, 52, 70, 83, 123). Mutational analysis suggested that stem-loop II is the major determinant for Rev interaction, consistent with the observation that stem-loop II is also critical for the Rev response in vivo (70, 83). Daefler et al. found that the first 90 nucleotides of the RRE are sufficient for direct Rev interaction (18). Although this sequence does not contain an intact stem-loop II, it may refold to a secondary structure which mimics the hammerhead configuration in this region (114a). Rev interacts with RRE as a multimer. It is possible that multimerization is necessary in Rev function (82).

Mechanism of Rev Regulation: Splicing versus Transport

As briefly alluded to above, the regulation of viral RNA expression is greatly influenced by the action of Rev. In the course of an infection, the first appearance of the smaller (1.8- to 2-kb) multiply spliced RNAs in the cytoplasm coincides with the synthesis of the regulatory but not structural proteins. Over a period (usually within 24 h) there is a shift in the accumulation of the larger (4.3- to 9.2-kb) unspliced or singly spliced mRNAs which provide the templates for production of the full complement of virus structural proteins. This pattern of temporal expression during infection has recently been described (60, 119). When it was first observed that *rev* mutants expressed only multiply spliced mRNA and failed to accumulate the full-length and singly spliced RNA transcripts in the cytoplasm, it was proposed that Rev differentially regulated the splicing of viral mRNA (26, 94). Whether such is the case is still controversial. Several studies showed that Rev can act on RRE-containing RNAs which lack functional splice sites (21, 27). However, the possible involvement of cryptic splice sites has not been ruled out. Studies on the expression of chimeric globin-RRE pre-mRNA transcripts suggest that HIV-derived spliced sites were necessary for Rev function and response (11). Furthermore, stable base-pairing of the 5′ donor site to U1 RNA was also found to be important for Rev activation (45). An alternative, but not mutually exclusive, role of Rev is at the level of nuclear export, where direct interaction of Rev and RRE could shunt RRE-containing mRNA from the nucleus to the cytoplasm. This is supported by findings that under some circumstances, the level of spliced and unspliced RNA in the nucleus remained unaffected by the presence or absence of Rev, but the cytoplasmic content of such mRNA was dramatically altered (27, 44, 69). In this context, a repressive sequence that is found in the *gag*, *pol*, and *env* coding regions and is termed the *cis*-acting repressive sequence (90) may specifically trap RNA in the nucleus. Taken together, these findings suggest that Rev may circumvent spliceosome-mediated nuclear retention by binding to the RRE and disrupting the formation of spliceosomes, resulting in more efficient export of RRE-containing RNA to the cytoplasm (Fig. 4).

Tat AND Rev AS TARGETS FOR ANTIVIRAL THERAPY

In Vitro Assays for Inhibitors

The requirement for both Tat and Rev during virus replication offers the potential for development of specific antiviral strategies against HIV infection. Inhibiting the function of Tat and Rev during the initial stages of replication would most probably halt the generation of infectious viral progeny and subsequently stop any further infection of the target cell population. Two in vitro bioassay systems which may potentiate the screening of inhibitors effective against Tat have been described (28, 41). Introducing Tat into an LTR-CAT indicator cell line preincubated with a potential inhibitor of Tat function may prove to be a model system for studying antiviral drugs which may interfere with *trans*-activation. The subsequent activation or repression of CAT gene expression thus provides a sensitive and rapid means of measuring the effectiveness of the drug on Tat *trans*-activation. A similar approach can be taken for Rev. A reporter gene construct containing RRE sequences and *cis*-acting repressive sequences would be dependent on a functional Rev protein for activity, and inhibitors could be screened with a stable cell line expressing the reporter gene and Rev.

Transdominant Mutants

The generation of a transdominant negative phenotype provides a novel approach for inhibiting the wild-type function of a gene within the cell (53). Evidence for this proposal has been previously shown with a transdominant phenotype of the herpes simplex virus type 1 *trans*-activator VP16 (36). Normally permissive cells expressing the transdominant phenotype of VP16 could effectively inhibit herpes simplex virus type 1 replication and were subsequently made refractory to infection. Transdominant *tat* and *rev* mutants have recently been described and shown to interfere with their respective wild-type functions (68, 84). The potential for the

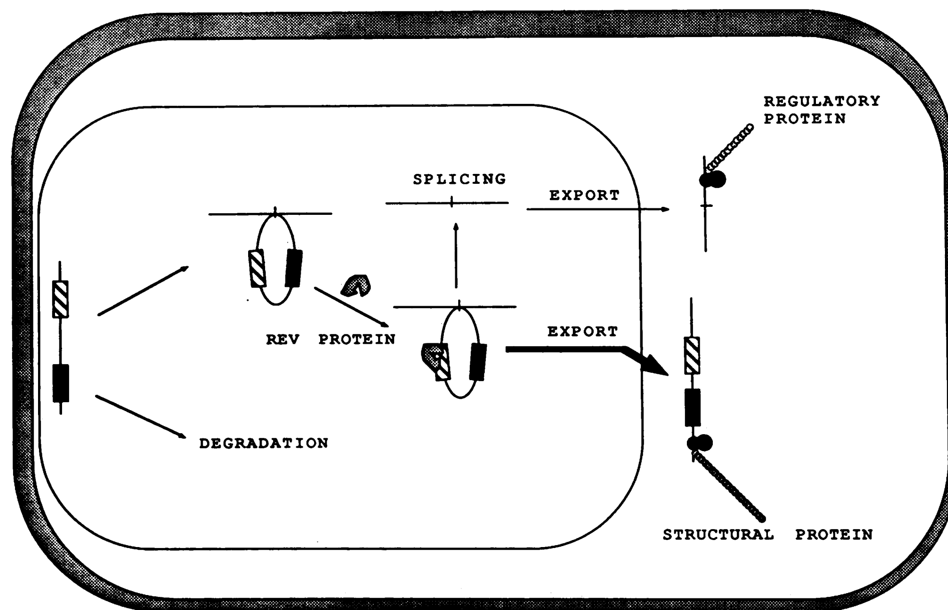

FIG. 4. Proposed model for *rev*-mediated *trans*-activation. RRE-containing mRNAs interact with Rev and are shunted from the nucleus to the cytoplasm (➡) at the expense of splicing (→). As a result, expression of structural proteins from the incompletely spliced mRNAs is differentially activated. Symbols: ▧, RRE; ■, *cis*-acting repressive sequence.

application of transdominant repression of HIV replication by these mutant alleles has been proposed. One such application of transdominant phenotypes can be envisioned in the following scenario. Hematopoietic stem cells isolated from the AIDS-infected individual are transfected with DNA or infected with a retrovirus vector containing either *tat* or *rev* transdominant alleles. Ideally, integration of the foreign DNA into the chromosomal DNA of a stem cell would result, and the expression of the mutant phenotype occurs at a level at which it is nontoxic to the metabolic functions of the cell. The genetically engineered stem cells expressing mutant Tat or Rev are then reintroduced into the patient's bone marrow, where they can repopulate to form a pool of cells resistant to HIV infection. The feasibility of such approaches would greatly depend on the latest developments in gene therapy, which is itself a rapidly advancing field.

Antisense Oligonucleotides

The use of antisense oligonucleotides as antiviral therapeutic agents is a relatively new approach to inhibit HIV infection. Antisense oligonucleotides derived from the initiation sequence of *rev* have been shown to be effective in blocking the expression of virus from chronically infected cells in a sequence-specific manner (72). Despite the effectiveness of these oligonucleotides as virus inhibitors in vitro, many other factors must come into play before practical application: pharmacokinetic properties, toxicity, and cost effectiveness, to name a few. So far, no clinical tests targeting oligonucleotides to HIV-infected cells in vivo have been performed.

EXPANDED REPERTOIRE OF Tat ACTIVITIES

A number of recent studies suggest that Tat function is not restricted to activation of virus gene expression in the infected cell, but may also affect uninfected cells through release and uptake and may activate genes from heterologous promoters. Additionally, Tat may modulate cell proliferation and contributes to the progression of at least one malignancy, Kaposi sarcoma.

Uptake of Tat

Purified Tat protein added to the extracellular culture medium is taken up by cells and is able to *trans*-activate LTR-directed gene expression (33, 41). Tat protein tagged by radioactivity or fluorescein conjugation binds to the cell surface in discrete regions and migrates to the nucleus and the nucleolus of treated cells (31a, 46a). Binding of labeled Tat to the cell is very rapid and is not inhibited by excess unlabeled Tat, suggesting that Tat enters the cell via endocytosis in clathrin-coated pits rather than a specific cell receptor.

Extracellular Tat

Tat protein is present in the medium of HIV-1-infected cells and cells which have been transfected with *tat* DNA (24). The amount of extracellular Tat observed under these conditions does not correlate directly with the amount of cell death. However, it cannot be entirely ruled out that the major source of extracellular Tat is derived from dying cells. Alternatively, Tat protein may be actively secreted. Although Tat lacks a consensus signal peptide, a number of cytokines, including IL-1, IL-1β, and the acidic as well as basic fibroblast growth factors and platelet-derived endothelial growth factors can be exported from cells in the absence of a known secretory consensus signal peptide. Tat may be an additional protein excreted from cells by a mechanism as yet unknown.

Transcellular Activation

Cocultivation of a T-cell line constitutively expressing HIV-1 Tat with cells that contain an integrated copy of an HIV-1 LTR reporter gene construct results in a significant increase in the activity of the reporter gene, indicating that Tat proteins produced by one cell can activate the HIV-1 LTR in adjacent cells (52a). Such transcellular activation requires cell-cell contact. Cocultivation of Tat-expressing cells with those containing a stably integrated *tat*-defective provirus was also able to rescue virus production (96).

Tat Binding to Cellular Adhesion Molecules

The carboxy terminus of Tat contains a highly conserved tripeptide sequence, Arg-Gly-Glu (RGD), which is characteristic of many extracellular matrix proteins which bind to cell adhesion molecules. Scanning of overlapping peptides derived from the *tat* sequence for their ability to bind cells revealed that a variety of cell types adhere to peptides containing the RGD sequence (62a). Experiments with intact Tat protein and competition by RGD-containing peptides confirmed that Tat may bind to a cell adhesion molecule (10). The biological consequence of this interaction is still unclear, but it is likely to have some impact on cellular functions. Thus, the second exon of *tat*, previously thought to be devoid of function, may have a role in viral pathogenesis.

Tat *trans*-Activates Heterologous Promoters

Although the HTLV *trans*-activator, Tax, is known to activate a number of cellular promoters besides the HTLV LTR, until recently Tat was thought to be highly specific for the HIV LTR. Recently one example is of Tat *trans*-activation of a heterologous promoter, the JC virus late promoter, has been reported (113). *trans*-activation of the JC virus promoter is observed only in glial cells, but not in T cells or fibroblasts; therefore, specific cellular factors present in microglial cells may be required for such activity. It is possible that this activity of Tat contributes to neurological disease by reactivation of latent JC virus. Replicating JC virus causes progressive multifocal leukoencephalopathy.

Tat as an Inhibitor of the Immune System

Purified Tat protein added to the culture medium is reported to inhibit antigen-induced proliferation of lymphocytes (116). The effect is specific for functional Tat, as oxidized and mutant proteins are inactive. The inhibitory effect of Tat on proliferation is itself inhibited by anti-Tat antibodies. The physiological significance of this observation remains unclear, as it is unlikely that circulating levels of Tat protein are sufficiently high to exert a general regulatory effect. If Tat does mediate such an effect, it probably does so in the context of generation of new immune cells.

Tat as a Growth Factor for Kaposi Sarcoma Cells

The incidence of Kaposi sarcoma is increased 1,000-fold in certain populations infected with HIV-1. The role of the HIV-1 virus in the disease must be indirect, as HIV-1 sequences are not found in the tumor cells. To study the relationship between infection and Kaposi sarcoma, cell lines were established from Kaposi sarcoma lesions of AIDS patients. These cell lines have several characteristics similar to the original tumor cell. The cells are spindle shaped and induce neoangiogenesis as well as tumors in nude mice. The cell lines were established by culturing tumor explants in medium conditioned with activated T-cells (mitogen-treated or HTLV-I-infected cells) or in the supernatant of HIV-1 infected cells (77, 97). The culture medium of HIV-infected cells also stimulates growth of the cultured Kaposi sarcoma-derived cells, but has no effect on growth of smooth muscle cells or endothelial cells, the putative progenitor of the Kaposi sarcoma tumor cells. Recently, it was shown that extracellular Tat protein present in the culture supernatant is the substance that provides growth stimulation, since this activity is blocked by anti-Tat antibodies (24). Moreover, purified Tat protein also stimulates growth of the Kaposi sarcoma-derived cell line in the depleted medium. Since the optimal threshold for this growth activity is much lower than that required for Tat protein-mediated LTR activation, the two phenomena may occur by different mechanisms. In addition, this low concentration of Tat may be attainable under physiological conditions. The inability of Tat to induce growth of the normal Kaposi sarcoma progenitor cells indicates that Tat protein is by itself unable to induce formation of Kaposi sarcoma. Tat protein may facilitate progression of this disease. The potential role of *tat* in the induction of Kaposi sarcoma is also supported by studies which indicate that mice carrying *tat* as a transgene developed lesions which some investigators think have a Kaposi tumor phenotype (117).

OPPORTUNISTIC VIRUSES AS COFACTORS

Many opportunistic viruses have been implicated with a role in the promotion of AIDS in the HIV-infected individual (79). Also, it has been suggested that viruses which cause severe opportunistic infections in AIDS patients may also serve as cofactors which initiate the onset of AIDS or accelerate the progression of the disease. The human T-leukemia viruses, HTLV-I and HTLV-II, which infect the same target cell as HIV, may play a role in reactivating HIV in the persistently infected T cell. Siekevitz et al. (105) and Böhnlein et al. (7) demonstrated that the HTLV-I *trans*-activator gene *tax* could activate the HIV LTR, exerting its effect indirectly through an inducible cellular factor which interacts with the NFkB-binding site located in the LTR enhancer region. Coincidently, activation of the IL-2 receptor α gene is also mediated through the interactions of these *tax*-inducible cellular factors with the NFkB-binding site located in the IL-2 receptor promoter region. This finding demonstrates the possibility of a unique overlap between two very different pathways of T-cell activation and HIV proliferation in infected T cells (6). The HIV-1 provirus, normally quiescent within the T cell, would now be activated by HTLV-I Tax-induced cellular proteins which are normally involved in T-cell proliferation.

Several other DNA viruses which may permit reactivation of the HIV provirus have been examined. Herpes simplex virus type 1 and Human herpesvirus 6 (HHV-6), now commonly found in the same population at risk for HIV, have been shown to increase viral gene expression from the HIV LTR in vitro (25, 65, 74). However, only HHV-6 shares the same tropism for the $CD4^+$ target cell as HIV, which suggests that HHV-6 might contribute to the $CD4^+$ depletion seen in AIDS patients. Lusso et al. (65) have demonstrated in vitro that $CD4^+$ death in doubly infected cells occurred at an accelerated rate when compared with that of $CD4^+$ cells

infected with HIV or HHV-6 alone. HHV-6 may increase the rate of HIV replication by acting directly on the HIV LTR, thereby increasing LTR-directed viral gene expression and subsequent cytopathic effect on the target cell population. However, it is not known whether the progression of AIDS increases in individuals coinfected with these viruses.

Another herpesvirus, human cytomegalovirus, has been detected in T lymphocytes during HIV infection (99). It has also been found along with HIV in the brain tissues of AIDS patients (80). Although human cytomegalovirus infection can lead to severe opportunistic disorders in AIDS patients, it is unclear whether the virus enhances HIV replication and $CD4^+$ depletion in HIV-infected individuals.

CONCLUSIONS

HIV exhibits a complex replication cycle which is tightly controlled at multiple levels. Two of the viral proteins, Tat and Rev, play key roles in this regulatory process. Although the virus genome contains a promoter which is relatively inefficient, Tat confers high-level expression by allowing more frequent initiation and more efficient elongation during transcription and may also facilitate translation of viral mRNA. The Rev protein, on the other hand, mediates a unique process of differential and temporal regulation of viral gene expression. Both proteins directly interact with their cognate RNA target sequences, bringing a new focus on RNA-protein interactions in gene regulation. The fact that *tat* and *rev* are essential genes for HIV replication has stimulated an intensive search for specific inhibitors, with some prospects of success. Although several of the other genes play more subtle roles in virus infection and transmission, inhibitors of their function could also ameliorate disease progression in infected individuals. Therefore, the genetic complexity of HIV may be ultimately turned to its disadvantage.

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