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Mechanism of Action of Regulatory Proteins Encoded by Complex Retroviruses

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INTRODUCTION

Retroviruses are named for their ability to reverse the normal flow of genetic information from genomic DNA to mRNA (214). Although retroviruses form a clearly defined and relatively homogeneous viral genus, they have nevertheless historically been subdivided into three taxonomic groupings primarily on the basis of the pathologic consequences of infection (204). The oncovirus subgroup includes retroviruses able to cause neoplastic disease in the infected host but also includes several related, apparently benign viruses (204, 214). Lentiviruses cause slow, chronic diseases that generally, but not always, lack a neoplastic component (78). Although the members of the spumavirus subgroup cause a marked foamy cytopathic effect in tissue culture, they have yet to be clearly associated with any human or animal disease (89, 222).

Because of the ability of the oncovirus subgroup to induce oncogenesis in experimental animals, research into retrovirus molecular biology has historically focused on this subgroup. These efforts led to the definition of the retrovirus life cycle visualized in Fig. 1. Retroviral replication initiates with the intracytoplasmic penetration of the virion core, a process mediated by the specific interaction of the viral envelope glycoprotein with a specific cell surface receptor (214). Subsequently, a virion-associated RNA-dependent DNA polymerase transcribes the single-stranded RNA genome into a double-stranded linear DNA proviral intermediate. This proviral intermediate then migrates to the nucleus, where a viral integrase covalently links the retroviral genome to host chromosomal DNA, thereby forming the retroviral provirus (214).

In its simplest form, as seen, for example, in murine leukemia virus (MLV), retroviral replication requires only three virally encoded genes (Fig. 1 and 2). These are the *gag* gene, which encodes the virion structural proteins, the *pol* gene, which encodes the various virion-associated enzymes, and the *env* gene, which encodes the envelope glycoprotein. (A viral protease, required for the posttranslational processing of the Gag and Gag-Pol polyproteins, may be encoded within *pol* or *gag*.) In the integrated DNA provirus, these three genes are invariably arranged in the same order (5′-*gag-pol-env*-3′) and are flanked by the characteristic long terminal repeats (LTRs) generated during the process of reverse transcription (Fig. 1 and 2). The LTRs contain enhancer and promoter elements required for transcription of the retroviral genome (214) and also contain sequences important for efficient mRNA polyadenylation (25). In MLV and most other animal oncoviruses, the integrated provirus encodes only two distinct transcripts. These are the genomic RNA, which also functions as the mRNA for Gag and Pol, and a singly spliced mRNA species that encodes Env (214). It therefore appeared that the life cycle of MLV and, by extension, that of retroviruses in general was both simple and efficient. Viral gene products served structural or enzymatic functions, while regulation of viral gene expression, be it at the transcriptional or posttranscriptional level, was controlled entirely by the interaction of *cis*-acting viral DNA or RNA sequences with *trans*-acting factors encoded by the host cell.

A resurgence of scientific interest in retroviruses, resulting particularly from the emergence of human T-cell leukemia virus type I (HTLV-I) and human immunodeficiency virus type 1 (HIV-1) as important human pathogens, has more recently led to the realization that the simple life cycle delineated above is not a fully adequate description of the replication cycle of all the members of this viral genus. For example, HIV-1 encodes no fewer than six gene products in addition to the characteristic retroviral Gag, Pol, and Env (Fig. 2), and these are translated from a novel set of singly spliced and multiply spliced viral mRNA species (42, 144, 147). At least two of these additional proteins, termed Tat and Rev, act in *trans* to directly regulate HIV-1 gene expression (42). Overall, although the steps between penetration and proviral integration appeared quite similar for both MLV and HIV-1, postintegration events were found to

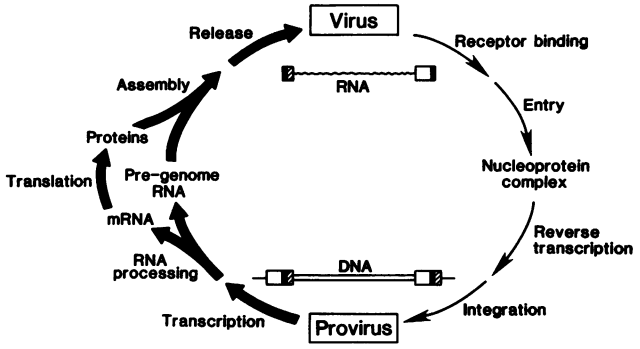

FIG. 1. Overview of the retrovirus replication cycle. Preintegration events are indicated by light arrows, while postintegration events are denoted by thick arrows. See the text for a discussion. Modified from reference 214 with permission.

be significantly more complex in the latter. More recently, it has become evident that HIV-1 is merely the most fully understood representative of a whole class of animal retroviruses that are now referred to as complex retroviruses. Retroviruses belonging to this category, which includes all lentiviruses and spumaviruses, as well as HTLV-I and related viruses (Table 1), are distinguished from MLV and other simple retroviruses not only by the greater complexity of their genomes (Fig. 2) but also, more particularly, by the pattern of viral gene expression that is displayed in the infected cell (41). This shared regulatory pattern, which both requires and facilitates a more complex genomic organization, is depicted in Fig. 3.

All integrated proviruses are acted upon by host cell transcription factors (214). For simple retroviruses, these DNA sequence-specific interactions are, in the appropriate cell context, fully sufficient to induce the production of a high level of proviral transcripts. However, whereas complex retroviruses remain dependent on the action of the host cell transcriptional machinery, these cellular factors are no longer sufficient to permit efficient viral gene expression (8, 33, 42, 67, 194, 195, 214). Instead, this interaction normally results in a low, basal level of viral mRNA synthesis. It has now been shown for several complex retroviruses that this initial population of genome length transcripts reaches the cell cytoplasm exclusively in the form of the small, multiply spliced mRNAs that encode the viral regulatory proteins (Fig. 3). The first of these early gene products to exert its effect is a virally encoded *trans*-activator of LTR-dependent transcription that is exemplified by the Tat protein of HIV-1 (Fig. 3) (56, 58, 81, 92, 131, 208). Because these viral transcriptional *trans*-activators efficiently enhance their own expression, they are able to induce a powerful positive-feedback loop (8, 33, 42, 67, 194, 195). This enhanced

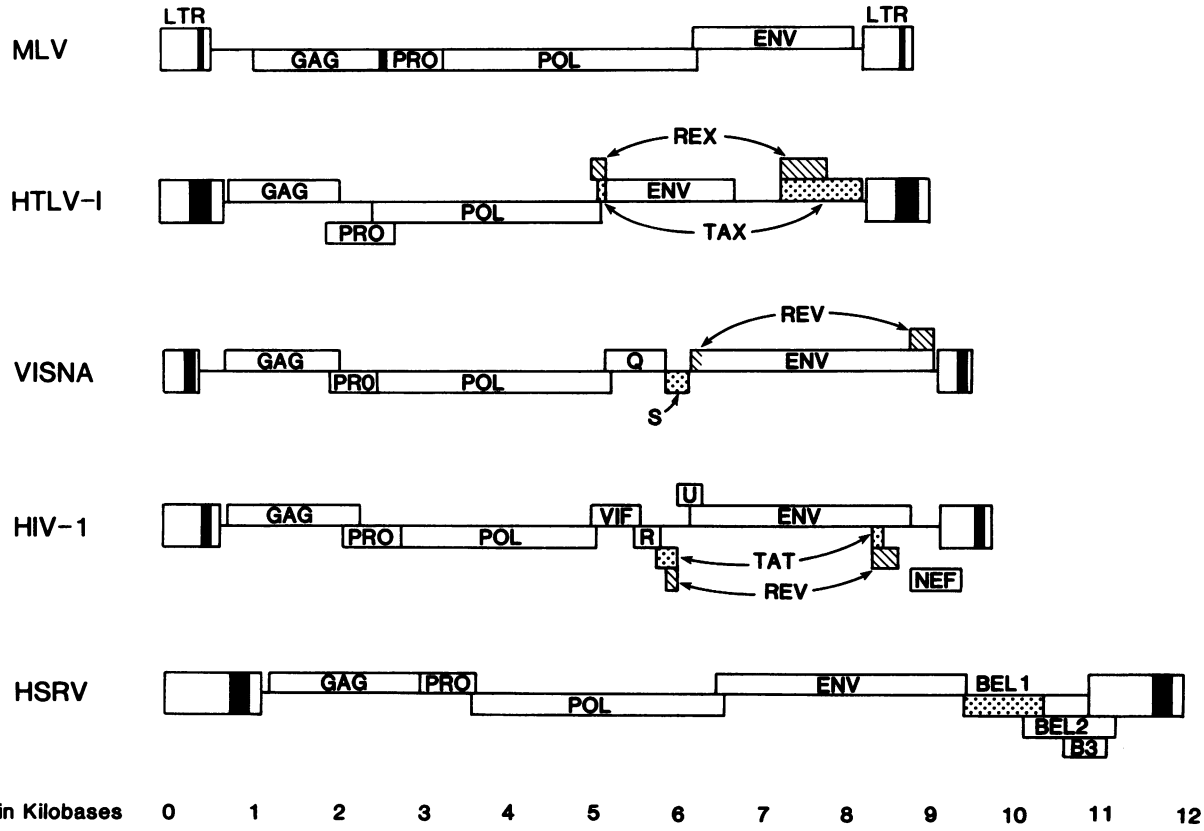

FIG. 2. Retrovirus genomic organization. A comparison of the proviral genomic organization seen in MLV, a representative simple retrovirus, with that seen in HIV-1 and other complex retroviruses. Although all known viral genes are named and drawn to scale, this should not be viewed as an exhaustive listing. Known transcriptional activators are marked by stippling, and known posttranscriptional regulators are indicated by hatching. LTRs are indicated by large terminal boxes with the "R" region in black. Abbreviations: R, Vpr; U, Vpu; B3, Bel-3. Reproduced from reference 41 with permission.

TABLE 1. Major taxonomic divisions among retroviruses

Category	Subgroup	Prototype[a]	Other examples[a]
Simple retroviruses	C-type retroviruses group A	RSV	ALV, ASV
	C-type retroviruses group B	MLV	FeLV, MSV, SNV, REV, SSV
	B-type retroviruses	MMTV	
	D-type retroviruses	MPMV	SRV-1
Complex retroviruses	Lentiviruses	HIV-1	HIV-2, SIV, visna virus, FIV, EIAV
	T-cell leukemia viruses	HTLV-I	HTLV-II, STLV, BLV
	Spumaviruses	HSRV	SFV, BFV

[a] Abbreviations: RSV, Rous sarcoma virus; ALV, avian leukemia virus; ASV, avian sarcoma virus; FeLV, feline leukemia virus; MSV, murine sarcoma virus; SNV, spleen necrosis virus; REV, reticuloendotheliosis virus; SSV, simian sarcoma virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; SRV-1, simian retrovirus type 1; STLV, simian T-cell leukemia virus; BFV, bovine foamy virus.

proviral transcription leads to the accumulation of a critical level of a second, posttranscriptional regulatory protein (termed Rev in HIV-1) that inhibits the further synthesis of the multiply-spliced regulatory mRNAs and instead activates the expression of the unspliced and singly-spliced mRNAs that encode the viral structural proteins (Fig. 3) (56, 58, 81, 92, 131, 208). This action divides viral gene expression into two temporal phases, i.e., an early, regulatory phase and a later, structural phase. The replication cycle of the complex retroviruses, as typified by HIV-1, is therefore distinguished from that of the simple retroviruses, as typified by MLV, by several criteria (Fig. 3). (i) Complex retroviruses encode a third, multiply spliced class of viral transcripts in addition to the singly spliced (Env) and unspliced (Gag-Pol) mRNA seen in the simple retroviruses. This early mRNA class encodes two nuclear regulatory proteins. (ii) The first of these regulatory proteins is a sequence-specific *trans*-activator of LTR-driven gene expression. (iii) A second regulatory protein is required to activate the expression of the late, structural mRNA species. (iv) The combined action of these two proteins divides the replication cycle of complex retroviruses into two temporal phases, a pattern not seen in the simple retroviruses.

Representative retroviral genomes drawn from each of the three subgroups of the complex retroviruses, i.e., the lentiviruses, the T-cell leukemia viruses, and the spumaviruses (Table 1), are shown in Fig. 2. These viruses each display a distinct genomic organization and have very limited primary sequence homology (147), yet they all appear to share the pattern of viral gene regulation depicted in Fig. 3 and can therefore be distinguished from MLV (Fig. 2) and other simple retroviruses on the basis of the criteria enumerated above. However, although each of these four complex retroviruses is known to encode both a transcriptional and a posttranscriptional regulatory protein (note, however, that a Rev equivalent has not yet been reported for human spuma retrovirus [HSRV; see below]), this does not imply that the mechanisms of action of these proteins are identical or even similar. Indeed, it appears that each of the transcriptional *trans*-activators encoded by these viruses exerts its effect by a distinct mechanism. In contrast, the posttranscriptional regulatory proteins appear to have retained very similar mechanisms of action during the evolutionary divergence of these retroviral families. In this review, I will summarize the current understanding of the mechanism of action of these retroviral regulatory proteins and discuss their potential role in facilitating viral replication and pathogenesis.

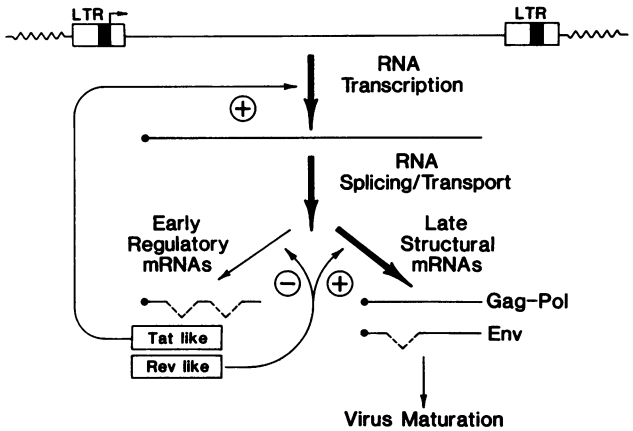

FIG. 3. Gene regulation in complex retroviruses. Complex retroviruses, including HIV-1, are defined by a pattern of gene regulation that requires the sequential action of a virally encoded "Tat-like" transcriptional activator and of a "Rev-like" posttranscriptional regulator. See the text for a detailed discussion. Reproduced from reference 41 with permission.

LENTIVIRUSES

HIV-1 is the primary etiologic agent of AIDS, a fatal disease that results from the gradual destruction of the helper T-cell population in infected individuals (12, 54, 137, 165). The importance of HIV-1 as a human pathogen has led to its being the major focus of research into lentivirus replication and gene regulation. Indeed, HIV-1 may be viewed as the prototype of not only the lentivirus subgroup but also, more broadly, complex retroviruses in general (41).

Tat Protein

Although the HIV-1 LTR contains binding sites for several cellular transcription factors, including the constitutively expressed protein SP-1 and the inducible factor NF-κB, the basal transcriptional activity of this promoter is quite low in most cellular contexts (69, 96, 97, 148). Expression of the viral Tat *trans*-activator results in a large, ~100-fold increase in HIV-1 LTR-dependent gene expression (8, 194). Both the functional expression of the viral *tat* gene product and an intact copy of the *cis*-acting target sequence for Tat, designated the *trans*-activation response element (TAR), are essential for HIV-1 replication in culture (46, 62, 174).

The *tat* gene is divided into two coding exons, which together predict the synthesis of an 86-amino-acid protein (Fig. 2). However, the first coding exon of Tat, which is flanked at its 3′ end by a conserved translation termination signal, is sufficient to encode a fully active, 72-amino-acid form of Tat (39). A singly spliced HIV-1 mRNA that encodes

this truncated form of Tat is, in fact, expressed late in the viral replication cycle (73, 130, 147). Three apparently distinct functional domains have been identified in Tat. A highly conserved motif containing seven cysteine residues has been proposed to bind metal ions and may mediate protein-protein interactions in vivo (64, 68). A C-terminal domain rich in lysine and arginine residues is required for the nuclear and nucleolar localization of Tat (Fig. 4) in expressing cells and will also direct heterologous proteins to the nucleolus when present in *cis* (84, 178, 190). This basic motif also serves as the RNA-binding domain of Tat. Between these domains lies a conserved "core" domain which has been proposed to function as the activation domain of Tat (30, 113).

TAR is a 59-nucleotide RNA stem-loop structure located at the 5′ end of all HIV-1 transcripts (83, 94, 145, 174) (Fig. 5). Both the location and orientation of TAR are critical for function (83, 174, 183). Mutational analysis has led to the hypothesis that the double-stranded RNA segments present in TAR serve primarily a structural role in the appropriate presentation of essential primary sequence information located in and immediately adjacent to the 6-nucleotide terminal loop and the 3-nucleotide bulge of TAR (18, 60, 83, 94, 177, 183). Direct evidence for TAR function at the RNA level has been obtained by the use of chimeric proteins consisting of Tat fused to a heterologous RNA-binding domain derived from the HIV-1 Rev protein (see below) or from the coat protein of the phage MS2 (184, 197). TAR was then replaced with the appropriate RNA target sequence (i.e., the RRE or the MS2 operator). In each case, the novel sequence specificity conferred on Tat by fusion to these heterologous RNA-binding domains was shown to permit transcriptional *trans*-activation of the HIV-1 LTR via the appropriate introduced target sequence, i.e., independently of TAR. This observation demonstrates that the only essential role for TAR is to act as the RNA target sequence for Tat.

Direct evidence that Tat possesses the ability to specifically bind TAR in vitro has been provided by several groups (29, 50, 176, 220). The basic domain of Tat has been shown to be both necessary and sufficient for this in vitro interaction (29, 220), thus placing Tat into the arginine-rich class of sequence-specific RNA-binding proteins (115). Mutations that disrupt the TAR stem structure or that affect the 3-nucleotide bulge have been reported to reduce both in vivo Tat function and in vitro Tat binding (Fig. 5) (18, 49, 60, 176, 177, 202). However, mutations of the 6-nucleotide terminal loop sequence of TAR that are equally deleterious in vivo have little or no effect on this in vitro interaction (18, 49, 60, 176, 177, 202). Although the subsequent demonstration that the in vitro interaction between Tat and TAR occurs entirely at the site of the small, pyrimidine-rich bulge (29, 49, 176, 202, 220) provided a biochemical explanation for this observation, it also strongly implied that this direct interaction, although necessary, was not sufficient to mediate the functional interaction of Tat with its RNA target in vivo. In contrast, for the chimeric Tat fusion proteins, binding to a heterologous RNA target sequence was fully sufficient for *trans*-activation (184, 197). Evidence indicating that a second, cellular factor is indeed involved in mediating the Tat-TAR interaction in vivo has recently been provided by research focused on the use of "TAR decoys" as inhibitors of HIV-1 replication (201). In these experiments, T cells were infected with retrovirus vectors that constitutively express very high levels of the 59-nucleotide TAR (Fig. 5) in the hope that this RNA would sequester any Tat protein expressed from an infecting HIV-1 provirus and, hence, block viral gene expression. This strategy was indeed found to result in the efficient protection of T cells in culture against challenge by HIV-1 (201). As predicted, TAR decoys lacking the appropriate RNA bulge sequence were unable to inhibit HIV-1 replication. Surprisingly, however, a mutation in the terminal loop that inhibited TAR function in vivo, but had no effect on Tat binding in vitro, also prevented any inhibition of HIV-1 replication in expressing cells. This observation is most easily explained by the hypothesis that this mutated TAR decoy was not capable of efficiently interacting with Tat in vivo despite an unimpaired in vitro interaction. It is therefore believed that the interaction of Tat with TAR in vivo requires the cooperative binding of a cellular RNA sequence-specific protein to the TAR terminal loop (Fig. 5). The hypothetical direct interaction of Tat with a cellular cofactor could, of course, occur prior to RNA binding. It is therefore possible that such a cellular Tat-binding (and TAR-binding) protein might well play a critical role in mediating Tat function not only through TAR but also through heterologous RNA target sequences.

Although several groups have reported cellular proteins capable of specifically interacting with either TAR or Tat itself, none of these factors have yet been persuasively shown to be critical for Tat function in vivo. However, at least two proteins have been reported to interact specifically with the terminal loop sequence of TAR, and hence they represent strong candidates for this hypothetical cellular cofactor (135, 187, 224). Clearly, a more complete understanding of Tat function requires the identification of this cellular factor and an analysis of its normal role in the regulation of cellular gene expression.

Many studies have shown that Tat increases the steady-state level of transcripts derived from genes linked to the HIV-1 LTR (39, 162, 223). There has also been general agreement that this increase results from an enhancement in the rate of transcription of such genes rather than from the stabilization of mRNAs containing TAR (85). However, the mechanism by which Tat enhances the rate of transcription has been more controversial. It has been suggested that Tat acts to increase the rate of transcription initiation, thus making TAR the RNA equivalent of a DNA enhancer sequence (114, 185, 198). Alternatively, it has also been proposed that Tat could function to prevent premature termination of transcripts initiated within the HIV-1 LTR (40, 99, 114, 168). The latter hypothesis originally derived from the observation that Tat had little effect on the level of RNA polymerase density adjacent to the site of transcription initiation but dramatically increased the rate of transcription of sequences distal to the HIV-1 LTR (99). It therefore appeared that transcription from the HIV-1 LTR was terminating prior to the complete synthesis of functional mRNA species, a phenomenon that has also been observed for some cellular proto-oncogenes (199). The hypothesis that Tat functions at least in part by promoting transcription elongation has now been confirmed both in vivo and in vitro (55, 103, 134). It appears that the transcription termination observed in the absence of Tat occurs at multiple, possibly random locations in viral or heterologous sequences linked to the HIV-1 LTR, thus suggesting that Tat acts by increasing processivity rather than by preventing a specific termination event (140).

The results discussed above imply that transcription complexes initiating in the HIV-1 LTR are only poorly able to elongate through adjacent DNA templates. This lack of processivity is somehow rectified by the interaction of the

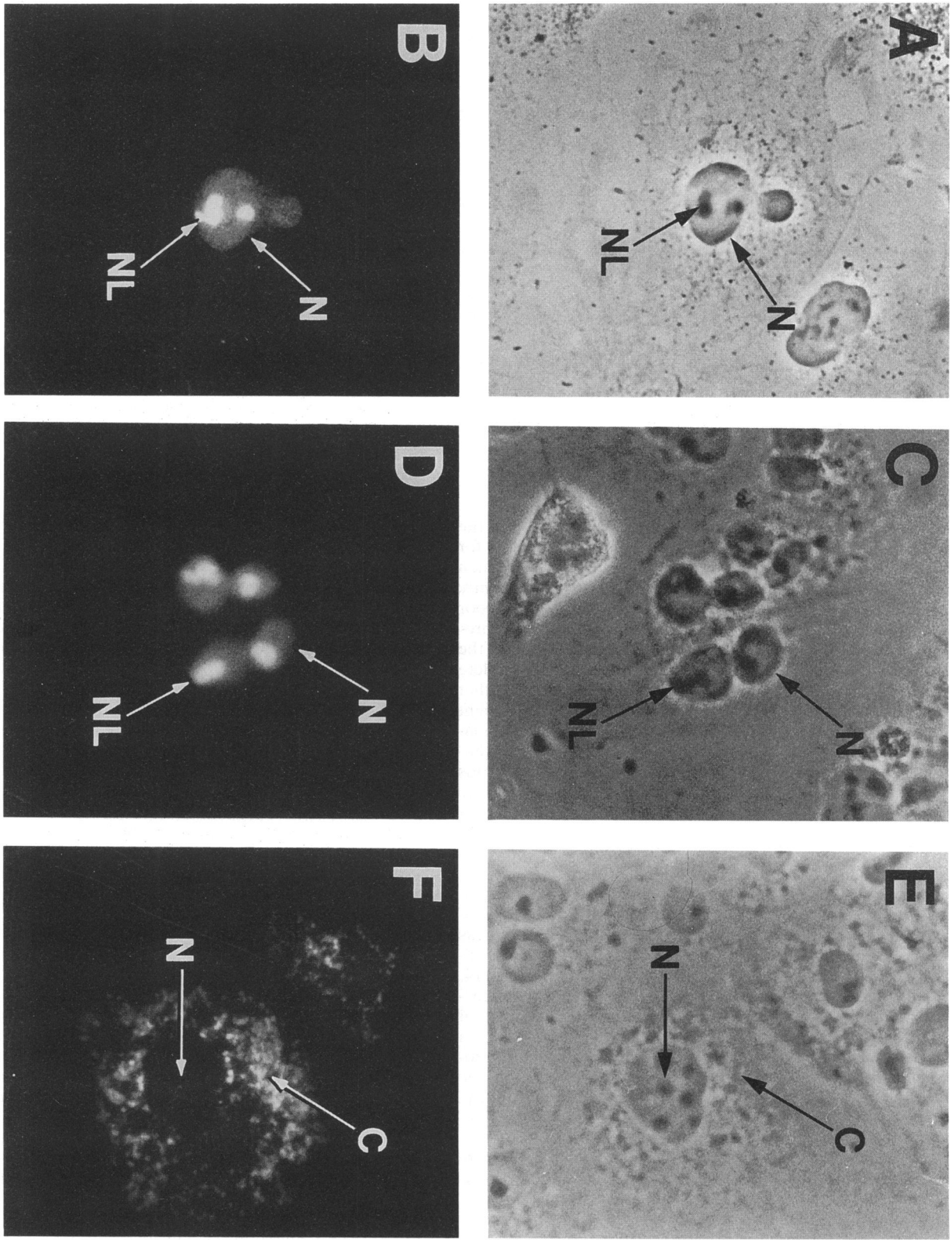

FIG. 4. Subcellular localization of the early gene products of HIV-1. Phase-contrast (A, C, and E) and immunofluorescence (B, D, and F) photographs of cells transfected with expression vectors that encode Tat (panels A and B), Rev (panels C and D), or Nef (panels E and F). Both Tat and Rev concentrate in the nuclei (N) and nucleoli (NL) of cells, whereas Nef is expressed exclusively in the cell cytoplasm (C).

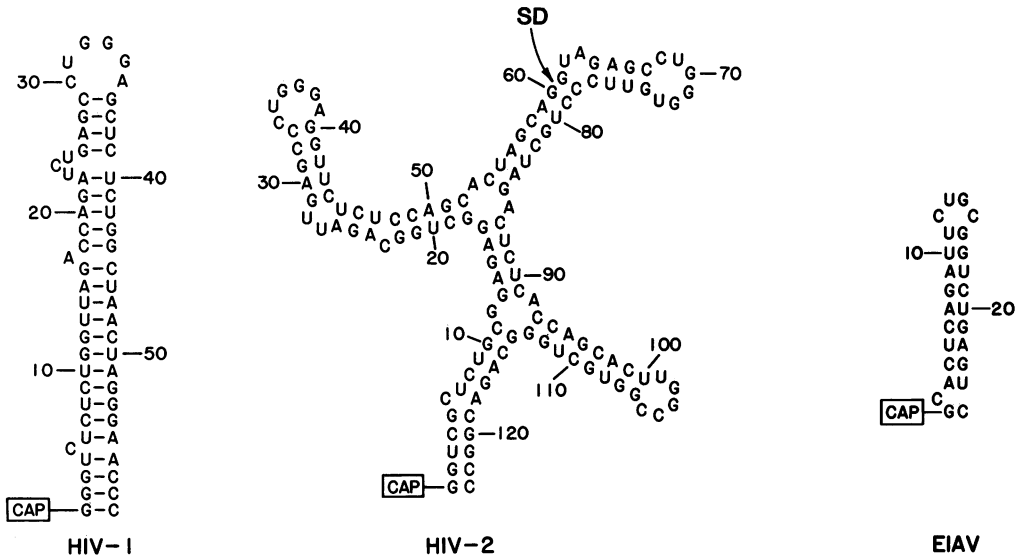

FIG. 5. Predicted RNA secondary structure of the lentivirus TARs. The TARs of the primate lentiviruses present primary RNA sequence information located in the terminal hexanucleotide loop (consensus 5'-CUGGGX-3'), and in the adjacent uridine-rich bulge, in an appropriately structured context. Little is known about EIAV TAR function. The splice donor (SD) indicated in the HIV-2 TAR element is functional in vivo (217).

Tat protein with the nascent TAR and, potentially, with the transcription complex itself (20, 40). Neither the reason for this premature termination nor the mechanism by which rescue of these transcription complexes occurs is currently understood. However, it does appear likely that this poor processivity must be encoded within the HIV-1 LTR promoter element itself (19, 168). It remains unclear whether the HIV-1 LTR simply lacks the ability to assemble a complete transcription complex or whether one or more factors that interact with LTR promoter sequences specify inefficient elongation. It is, however, of interest that Tat, in combination with TAR, can at least modestly *trans*-activate transcription from such standard promoters as the LTR of Rous sarcoma virus, the simian virus 40 early promoter, and the cytomegalovirus immediate-early promoter (39, 174). It therefore seems possible that the poor processivity observed during basal HIV-1 LTR-driven transcription is simply an extreme example of a relatively general phenomenon.

Although results from several groups support the hypothesis that transcriptional *trans*-activation by Tat results primarily from enhanced transcription elongation, evidence has been presented suggesting that Tat can also act to significantly increase the level of transcription initiation (103, 114, 198). It therefore appears possible that the increased processivity of transcription complexes formed in the presence of Tat is correlated with an increased ability to assemble a functional transcription complex at the HIV-1 LTR promoter (114). Whether Tat affects mRNA synthesis at the level of both initiation and elongation and, more importantly, perhaps, whether these two phenomena are indeed functionally interlinked (103, 114, 198) remains to be fully resolved. The recent demonstration of an in vitro transcription system that appears to faithfully reproduce TAR-dependent *trans*-activation of the HIV-1 LTR by Tat should represent a key step toward the eventual unraveling of the mechanism of action of this novel regulatory protein (134).

Although activation of viral RNA transcription is the major action of Tat in most experimental systems, it is clearly not the only effect of this small *trans*-activator. A number of reports have noted that the effect of Tat on the level of expression of genes linked to the viral LTR, when measured at the protein level, can be significantly higher than the effect determined at the level of steady-state mRNA (13, 39, 56, 173, 223). Although the molecular basis for this second, posttranscriptional component of the bimodal action of Tat remains unclear, it also appears to be mediated by the sequence-specific interaction of Tat with the viral TAR RNA (27, 28). Results obtained from microinjection of TAR-containing RNA molecules into *Xenopus* oocytes show that this posttranscriptional effect also occurs in the cell nucleus yet can be segregated from the transcriptional action of Tat (27, 28). Thus far, Tat has not been shown to modulate the nuclear export of TAR-containing RNA species, and hence a more complex mechanism of action appears likely. One hypothesis suggests that Tat could affect the cytoplasmic compartmentalization, and hence the translational utilization, of TAR-containing transcripts by inducing a covalent modification of TAR-containing mRNA species that influences their cytoplasmic fate (28).

Rev Protein

As noted above, HIV-1 shares with other complex retroviruses the property of encoding two classes of viral mRNAs that can be distinguished on the basis of their temporal expression (Fig. 3) (41). An early class of viral transcripts consists of the multiply spliced, ~2-kb mRNA species that encode the viral regulatory proteins Tat and Rev and a third protein termed Nef. The late class of viral mRNAs consists of the unspliced (~9-kb) and singly spliced (~4-kb) transcripts that encode the virion structural proteins Gag, Pol, and Env and the auxiliary proteins Vif, Vpr, and Vpu (Fig. 2) (56, 73, 110, 130, 147). In the absence of functional Rev protein, only the fully spliced class of HIV-1 mRNAs is expressed (56, 130, 180, 193). Rev mutants of HIV-1 are incapable of inducing the synthesis of the viral structural

proteins and are therefore replication defective (180, 205). An analysis of the time course of HIV-1 infection of human T lymphocytes reveals a similar phenomenon (106). Initially, only the 2-kb class of viral mRNAs is detected in the cytoplasm of HIV-1-infected cells; however, as the level of viral gene expression increases (owing to the action of the Tat protein), a switch to the synthesis of the ~4- and ~9-kb viral mRNA species is observed. This effect, which reflects the action of the viral Rev *trans*-activator, occurs concomitantly with an essentially equivalent reduction in the synthesis of the fully spliced mRNA species that encode the viral regulatory proteins (56, 106, 130, 180). Therefore, Rev functions as a negative regulator of its own synthesis and also mediates the establishment of an equilibrium between viral structural and regulatory protein synthesis (130).

The switch from the early, regulatory phase of HIV-1 gene expression to the late, structural phase appears to require the expression of a critical level of Rev (141, 164). Several cell lines nonproductively infected with HIV-1 have been shown to constitutively express a low level of viral mRNA that is primarily of the 2-kb class. Treatment of these cells with agents that result in activation of the HIV-1 LTR also induces the expression of viral structural proteins (141, 164). It is therefore hypothesized that latency in this context is due to the expression of a subcritical level of the Rev *trans*-activator, a level which in turn reflects a paucity of cellular transcription factors critical for efficient HIV-1 LTR-dependent gene expression (71, 164). The Rev regulatory pathway may therefore serve to prevent the premature progression of the viral replication cycle to the late or lytic phase in cells that are incapable of supporting the required level of viral mRNA and protein synthesis (71).

Although Rev is absolutely required for the functional expression of unspliced HIV-1 RNA species, it appears to have little effect on the pattern of HIV-1 RNA expression in the cell nucleus (53, 58, 79, 131, 132). In particular, high levels of unspliced viral transcripts can be detected in the nucleus even in the absence of Rev. Although it appears evident that the splice sites present in HIV-1, like the splice sites present in other retrovirus transcripts, are inefficiently used by the cell-splicing machinery (6, 32, 100, 131), it has been less clear why these incompletely spliced viral transcripts remain sequestered in the cell nucleus in the absence of Rev. One hypothesis suggests that the viral structural genes might contain multiple copies of a *cis*-acting repressive sequence which functions to retain these RNAs in the nucleus in the absence of Rev (35, 53, 126, 175). An alternative hypothesis argues that the intact splice sites present in these incompletely spliced mRNAs act as nuclear retention signals (32, 122, 131). It has been suggested that splicing factors may be able to assemble on the primary HIV-1 transcript but that the subsequent splicing steps are performed only very slowly. Instead, this interaction results in the prolonged retention of these incompletely spliced viral transcripts within the nucleus (32, 122, 137). Persuasive evidence indicating that the U1 small nuclear ribonucleoprotein particle, a factor critically involved in the commitment of mRNA species to the splicing pathway (142), directly interacts with Rev-responsive HIV-1 mRNA species in vivo has been presented (122). Rev might therefore activate the nuclear export of these sequestered RNAs either by antagonizing their interaction with these splicing factors (32, 109, 122) or by directly facilitating their interaction with a component of a cellular RNA transport pathway (53, 58, 131). In contrast, in the absence of Rev, viral mRNAs are eventually fully spliced before being transported to the cytoplasm.

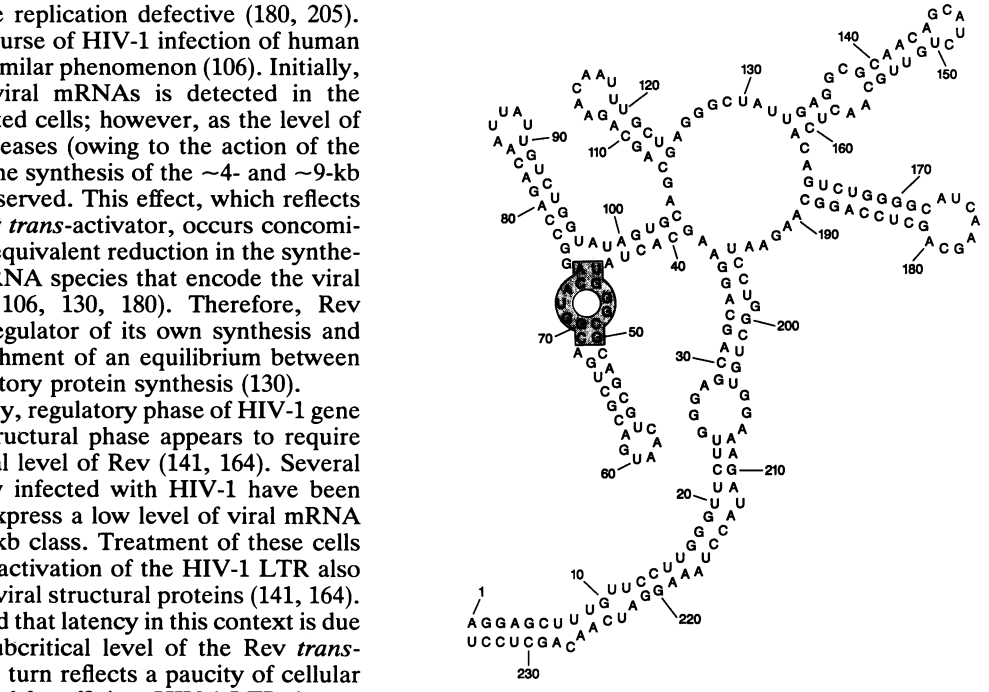

FIG. 6. Predicted RNA secondary structure of the RRE of HIV-1. The structured 13-nucleotide RNA sequence that forms the primary Rev-binding site is shaded.

The action of the Rev *trans*-activator is specific for unspliced HIV-1 transcripts and thus far has not been shown to affect the splicing or transport of cellular RNAs. The specificity of this response is conferred by a highly structured 234-nucleotide RNA target sequence, the Rev response element (RRE), that is located within the HIV-1 envelope gene (131, 174) (Fig. 6). The Rev protein has been shown to bind to the RRE with high affinity in vitro (43, 44, 86, 133, 159, 226). Although the entire 234-nucleotide RRE is required for full biological activity in vivo (91, 131), it appears that the RNA target sequence required for in vitro Rev binding consists of a structured 13-nucleotide sequence element that is contained within the full-length RRE (Fig. 6) (14, 86, 211). It is thought that the remainder of the RRE functions to stabilize the RNA structure of the primary Rev-binding site or to facilitate the presentation of this RNA sequence in vivo, or both (14, 86, 91, 211).

The *rev* gene consists of two coding exons that together predict a protein of 116 amino acids. Rev is localized to the nuclei and, particularly, the nucleoli of expressing cells (Fig. 4) (58, 128). Rev is phosphorylated at two serine residues in vivo; however, this posttranslational modification does not appear to be essential for Rev function (82, 128). Two distinct protein domains that are essential for Rev function have been defined (Fig. 7). The first is a ~50-amino-acid N-terminal sequence characterized by an arginine-rich central core that has been shown to function as the Rev protein nuclear/nucleolar localization signal (16, 36, 112, 128). This sequence is also both necessary and sufficient for the sequence-specific interaction of Rev with the RRE (16, 109, 129). Flanking this arginine-rich sequence are amino acid residues involved in the multimerization of Rev, a process which appears critical for in vivo Rev function (129, 158, 227). Mutations within any part of this sequence element

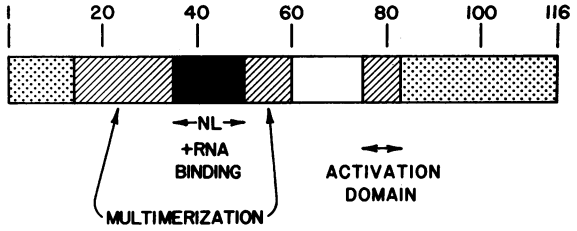

FIG. 7. Domain structure of the HIV-1 Rev protein. The RNA-binding domain of Rev contains a basic, arginine-rich core which is essential for both the nuclear localization (NL) of Rev and sequence-specific binding to the RRE. Rev function in vivo also depends on sequences immediately adjacent to the arginine-rich motif that are critical for multimerization of Rev on the RRE. The "activation domain" of Rev, a leucine-rich motif located toward the carboxyl terminus, plays no role in the interaction of Rev with the RRE. However, Rev proteins lacking a functional activation domain display a *trans*-dominant negative phenotype in vivo. The amino- and carboxy-terminal sequences indicated by stippling appear dispensable for Rev function. Reproduced from reference 129 with permission.

result in Rev proteins displaying a recessive negative phenotype (128, 158).

It seems highly probable that Rev also contains a protein domain that interacts directly with a component of the nuclear RNA transport or splicing machinery (132). Mutational analysis has suggested that a leucine-rich sequence centered on amino acid 80 serves this function (128, 132, 140, 158, 215). Rev proteins mutated in this activation domain retain full RRE binding and multimerization activity, yet not only are defective but also inhibit wild-type Rev function in *trans* (128, 132, 140, 158, 215). It has been proposed that these mutant Rev proteins inhibit Rev function by recruiting wild-type Rev protein molecules into mixed multimers that are compromised in their ability to interact with this cellular cofactor (129, 132, 158). Clearly, the definition of the in vivo target of this leucine-rich activation domain will be critical to the full resolution of the mechanism of action of Rev.

As shown in Fig. 4, both Tat and Rev are observed to concentrate in the nuclei and, particularly, the nucleoli of expressing cells. The significance of this nucleolar concentration has been obscure, given the very different functions of these two RNA sequence-specific regulatory proteins in the HIV-1 replication cycle. A possible explanation for this curious coincidence may be provided by the observation that the mutationally defined minimal sequence within Tat that is sufficient to confer in vitro affinity for the TAR RNA, i.e., the arginine-rich motif, is identical to the minimal sequence that is sufficient to act as an in vivo nucleolar localization signal when attached to heterologous cytoplasmic proteins (29, 190, 220). Similarly, the basic domain of Rev is both necessary and sufficient for localization to the nucleolus and specific binding to the RRE (16, 109, 112). It therefore appears that the nucleolar localization of both Tat and Rev may simply be a surrogate marker for the relatively nonspecific affinity of these proteins for structured RNA. In overexpressing cells, this property results in the concentration of Tat and Rev within the nuclear region that contains the highest concentration of structured RNA, i.e., the nucleolus. The observation that mutations of the arginine-rich motif that affect nucleolar localization also inhibit the in vivo function of Tat and Rev may therefore simply reflect the simultaneous inhibition of both the specific and the "nonspecific" RNA-binding properties of these proteins (16, 36).

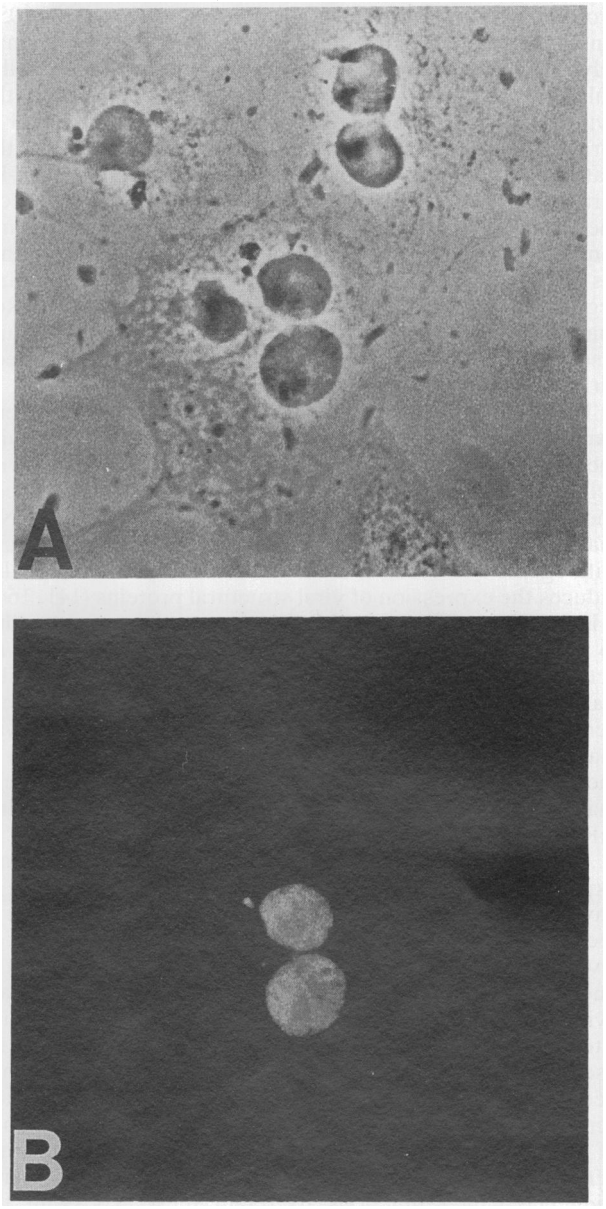

FIG. 8. Subcellular localization of the visna virus transcriptional *trans*-activator S. Phase-contrast (A) and immunofluorescence (B) photographs of cells transfected with the visna virus S gene expression vector pcS (208). The S *trans*-activator displays a diffuse nuclear fluorescence. The anti-S anti-peptide antibody used in these experiments was raised in rabbits as previously described (208).

As predicted by this hypothesis, both visna virus Rev and HTLV-I Rex are also observed to concentrate in the nucleoli of expressing cells (191, 208). In contrast, DNA sequence-specific retroviral *trans*-activators either display a diffuse nuclear fluorescence (visna virus "S") (Fig. 8) or are excluded from the nucleoli (HTLV-I Tax and HSRV Bel-1) (59, 102).

Other HIV-1 Auxiliary Proteins

In addition to Tat and Rev, HIV-1 encodes a third early-gene product termed Nef. Nef is a myristylated phosphopro-

tein that is associated with cytoplasmic membrane structures and excluded from the cell nucleus (Fig. 4) (80). Nef has been reported to possess the GTPase, autophosphorylation, and GTP-binding properties typical of the G-protein family of signal transduction proteins (77); however, this observation has not been confirmed (98, 152). Unlike Tat and Rev, the Nef gene product is not required for HIV-1 replication in culture. It has, in fact, been proposed that expression of Nef can result in an inhibition of HIV-1 LTR-specific gene expression and viral replication (2, 34, 123, 155, 206). However, these negative effects of Nef remain controversial, because other investigators have observed no effect of the Nef protein on either viral replication or gene expression (9, 80, 107). More recently, it has even been suggested that Nef can significantly enhance the replication of certain HIV-1 isolates in culture (207). Although the role of the *nef* gene product in the HIV-1 replication cycle therefore remains unclear, it does not appear likely that Nef acts by regulating the level of viral gene expression. One possibility is that Nef interferes with T-cell function by down-regulating CD4 expression or by interfering with signal transduction from the T-cell receptor complex (70, 124). Clearly, the fact that the Nef open reading frame is reasonably conserved in all primate lentiviruses (147) suggests that this protein is likely to play a significant role in the viral life cycle in the infected host. This hypothesis is strongly supported by the observation that a functional *nef* gene product markedly enhances viral replication and pathogenicity in rhesus macaques infected with a cloned isolate of simian immunodeficiency virus (SIV) (104).

In addition to Gag, Pol, and Env, the late gene products encoded by HIV-1 include the auxiliary proteins Vif, Vpu, and Vpr. Both Vif and Vpu play a role in the maturation and release of infectious HIV-1 virion particles (see reference 42 for a review). Vpr is detectable in the virion itself, i.e., is a structural protein, and modestly enhances HIV-1 replication rates in culture (37, 38, 157, 160). Although it has been proposed that Vpr can enhance HIV-1 LTR-dependent gene expression two- to fourfold, this result has proven difficult to reproduce (160). None of the late proteins of HIV-1 can therefore currently be defined as regulatory.

Regulatory Proteins of HIV-2 and SIV

Three subfamilies of primate and simian immunodeficiency virus have thus far been identified that are evolutionary related to, but distinct from, HIV-1 (147). The most intensely studied of these is HIV-2, a pathogenic human virus closely related to the rhesus macaque (SIVmac) and sooty mangabey (SIVsmm) isolates of SIV. Considerably less well studied are the distinct African green monkey (SIVagm) and mandrill (SIVmnd) subfamilies of SIV.

Although the regulatory proteins of HIV-2 and the related SIVmac and SIVsmm appear generally similar to HIV-1 Tat and Rev, several interesting differences are known to exist. For example, the TAR of HIV-2 is distinct in that this larger RNA structure contains two functional Tat target sites (17, 61) (Fig. 5). These each consist of a uridine-rich RNA bulge Tat-binding site positioned immediately 5′ to a terminal loop that conforms to the functional sequence established for HIV-1 TAR. Mutational analysis of the HIV-2 TAR has confirmed that both these Tat target sites indeed contribute to HIV-2 TAR function in vivo, although the 5′-proximal target site is clearly the more significant in conferring maximal Tat inducibility (17, 52, 61). The HIV-2 and SIVmac TARs have also been shown to provide fully functional targets for *trans*-activation by HIV-1 Tat (7, 17, 52, 61, 217). In contrast, both HIV-2 and SIVmac Tat are only partly active on the HIV-1 TAR (7, 17, 52, 61, 217). Although the molecular basis for this incomplete reciprocity remains unclear, it appears likely that it must reflect the inefficient interaction of these proteins with the heterologous HIV-1 TAR (17, 61).

An interesting aspect of the HIV-2/SIV TAR is that it contains a 5′ splice donor sequence that is used during the viral replication cycle (217) (Fig. 5). However, because Tat acts at the nascent RNA level (20), this site would not be predicted to have any effect on the Tat-TAR interaction. No equivalent splice site is observed in HIV-1, and the biological significance of this processing event is therefore unclear.

The RRE of HIV-2, like the RRE of HIV-1, coincides with a highly conserved RNA-folding region located within the viral *env* gene (120, 127). A computer-predicted RNA structure for the HIV-2 RRE has been proposed and not only is similar to the HIV-1 RRE but also is predicted to be highly conserved in the entire HIV-2/SIVmac/SIVsmm subgroup (116, 127). Both HIV-2 and HIV-1 Rev have been shown to specifically bind the HIV-2 RRE in vitro (47), and the HIV-1 Rev protein displays full biological activity when tested on the HIV-2 RRE (47, 120, 127). In contrast, the HIV-2 Rev protein is severely impaired in its ability to rescue viral structural protein expression through the HIV-1 RRE sequence (47, 120, 127). Analysis of the in vitro binding of HIV-2 Rev to the HIV-1 RRE has suggested that this protein-RNA interaction may be impaired in comparison with the binding of HIV-2 Rev to its cognate RRE (47, 72). Similarly, the Rev proteins of SIVmac, SIVagm, and SIVmnd are also unable to function via the HIV-1 RRE, although HIV-1 Rev is fully active on the RRE of SIVmac and SIVagm and at least partly active on the RRE of SIVmnd (127, 181). Presumably, these observations again reflect the degree to which these Rev proteins are able to functionally interact with the various viral RRE target sites.

Although the HIV-1 Rev protein has been subjected to intense mutational analysis, relatively little is known about the Rev proteins of HIV-2 and SIV (48). However, the amino acid sequences within HIV-2 Rev that are most highly conserved relative to the HIV-1 Rev protein coincide closely with the known HIV-1 Rev functional domains (Fig. 7). A conserved arginine-rich motif is believed to function as the nuclear/nucleolar localization signal and as the sequence-specific RNA-binding motif of HIV-2 Rev (48, 72). Also highly conserved is the leucine motif that is believed to function as the activation domain of HIV-1 Rev (132). Recently, it has been demonstrated that this HIV-2 sequence can indeed be substituted in place of the essential HIV-1 Rev leucine motif to yield a functional chimeric Rev protein (72).

Regulatory Proteins of Animal Lentiviruses

In addition to the relatively cohesive primate immunodeficiency viruses, the lentivirus family includes a somewhat more diverse group of nonprimate lentiviruses that infect primarily ungulates (78). The prototype of these nonprimate lentiviruses is visna virus, first described as the etiologic agent of a chronic degenerative syndrome observed in sheep (78). Other significant nonprimate lentiviruses include equine infectious anemia virus (EIAV) and feline immunodeficiency virus (FIV). Both visna virus and EIAV

represent economically significant livestock pathogens, whereas FIV is an increasingly important pathogen of domestic cats.

Although HIV-1 and visna virus share only limited primary sequence homology (147, 196), they do share the characteristic lentivirus genomic organization, marked particularly by the presence of additional open reading frames between the viral *pol* and *env* genes (Fig. 2). As with HIV-1, these genes have been found to encode the regulatory proteins that are typical of the complex retroviruses.

The S open reading frame of visna virus encodes a nuclear protein (Fig. 8) that is able to *trans*-activate visna virus LTR-dependent transcription (45, 74). Depending on the cell type, *trans*-activation levels of from as low as 3-fold to as high as 25-fold have been reported. The limited effectiveness of the S *trans*-activator may in part reflect the high basal level of transcription observed with the visna virus LTR in most cell types (73, 74). The DNA target sequences for the visna virus S protein are located entirely within the LTR U3 region and are homologous to the target sites of the cellular transcription factors AP-1 and AP-4 (87). Although these observations appear to imply an indirect mechanism of action for visna virus S protein, this question currently remains unresolved. Although the function of visna virus S protein, i.e., enhanced LTR-dependent transcription, is therefore analagous to that exerted by HIV-1 Tat, it appears that the target specificity and the mechanism of action of S protein are distinct. As perhaps predicted by this consideration, the visna virus S and HIV-1 Tat proteins also display little sequence homology (196). Overall, it therefore appears inappropriate to term this gene product visna virus Tat, as has been suggested elsewhere (45).

Visna virus is known to express the proviral genome in a temporally regulated manner (218). As predicted by this observation, visna virus has been shown to encode a nuclear protein that is functionally and mechanistically remarkably similar to the Rev protein of HIV-1 (208–210). In particular, visna virus Rev is required for the cytoplasmic expression of the singly spliced and unspliced mRNA species that encode the viral structural proteins (208). The *cis*-acting target sequence for visna virus Rev is a highly structured 176-nt RNA sequence, again located within the viral envelope gene (182, 209). Visna virus Rev binds to the visna virus RRE with high affinity in vitro but is unable to bind the HIV-1 RRE (209). This observation provides a biochemical explanation for the inability of visna virus Rev to rescue a Rev-defective HIV-1 provirus when present in *trans*. Despite this difference in sequence specificity, visna virus Rev appears to have retained a domain organization identical to that observed in HIV-1 (Fig. 7). Visna virus Rev has, in particular, been shown to retain both an arginine-rich motif that mediates sequence-specific RNA binding and a leucine-rich activation domain very similar to that observed in HIV-1 Rev (210). Indeed, chimeric proteins containing the binding domain from one Rev protein attached to the activation domain of the second are fully active on the RRE cognate for the RNA-binding domain (210). It therefore appears that the mechanism of action of Rev has been tightly conserved during the evolutionary divergence of HIV-1 and visna virus. In contrast, as noted above, visna virus and HIV-1 have evolved distinct strategies to activate transcription from their homologous LTRs.

Although EIAV is believed to be more closely related to visna virus than to HIV-1 (147), the transcriptional *trans*-activator encoded by EIAV appears to be mechanistically similar to HIV-1 Tat. In particular, EIAV Tat has been reported to act via a short RNA stem-loop structure located at the 5′ end of all EIAV transcripts (Fig. 5) (31). In addition, EIAV Tat displays clear amino acid homology to HIV-1 Tat, particularly in the Tat core and basic domains (51), and certain chimeric proteins derived from these two distinct viral *trans*-activators have been shown to retain biological function (30). However, the EIAV TAR does not display the sequence characteristics known to be essential for HIV-1 TAR function, and, presumably as a result, these proteins fail to cross-*trans*-activate. The lack of homology between the terminal loops of the HIV-1 and EIAV TARs may imply that these proteins interact with distinct cellular cofactors or that EIAV does not require such a cofactor. However, it has yet to be demonstrated that EIAV Tat can indeed specifically bind its proposed RNA target site either alone or in combination with a cellular protein.

The existence of an EIAV protein equivalent to Rev was initially inferred from the temporal regulation of viral gene expression observed in infected cells (167). Analysis of viral mRNA species expressed in infected cells led to the identification of a candidate Rev gene product (200). Recently, this protein has indeed been shown to activate EIAV-specific structural protein expression in *trans* (160).

Although FIV is attracting increasing attention as a model system for the study of virus-induced immunodeficiency, relatively little is known about the regulatory proteins encoded by this virus. However, recent data have demonstrated the existence of a Rev activity of FIV-infected cells and have assigned this activity to a specific viral open reading frame (108, 163). FIV Rev, like HIV and visna virus Rev, appears to act through a structured RNA target sequence located within the viral envelope gene to induce the expression of viral structural proteins (108, 163). Overall, these data suggest that a gene product functionally equivalent to HIV-1 Rev will be a general characteristic of lentiviruses.

T-CELL LEUKEMIA VIRUSES

Tax Protein

HTLV-I is the etiologic agent of adult T-cell leukemia, a generally fatal cancer that affects helper T cells (229). However, adult T-cell leukemia is generally of clonal origin and develops in only a small percentage of HTLV-I-infected patients and after a lag period of many years (229). Although adult T-cell leukemia is therefore likely to be the end result of a series of oncogenic events, it is nevertheless believed that the HTLV-I Tax protein, a nuclear *trans*-activator of HTLV-I LTR-dependent transcription (59, 195), plays a critical role in initiating this transformation process (229). Recently, it has been directly demonstrated that Tax can indeed facilitate cellular transformation in vitro (76, 203). The 40-kDa Tax *trans*-activator may therefore be viewed as functionally analogous to regulatory proteins encoded by several DNA tumor viruses including, for example, simian virus 40 T antigen and adenovirus E1A (33).

The viral DNA target sequence for Tax coincides with a set of three 21-bp repeats located within the HTLV-I LTR U3 element (66, 95, 161, 188). The invariant core of these repeats, the pentanucleotide 5′-TGACG-3′, is identical to the consensus binding site for the CREB/ATF family of cellular DNA-binding proteins (15, 95, 156, 225, 228). Several distinct members of this family of leucine zipper motif

proteins have been shown to specifically interact with the HTLV-I 21-bp repeat in vitro (156, 225).

Several lines of evidence suggest that Tax does not bind its DNA target sequence directly. In particular, purified recombinant Tax protein is not capable of directly interacting with its target site in vitro, whereas the pattern of protein binding to the 21-bp repeat sequence is not evidently affected by the expression of Tax (156). These data led to the hypothesis that the 21-bp repeats were instead bound by a consitutively expressed member(s) of the cellular CREB/ATF family (136, 156, 225). *trans*-activation by Tax would then require and reflect the direct interaction of Tax with one or more of these bound cellular proteins. Evidence in support of this model has now been presented by several groups (15, 136, 228), although the precise identity of the cellular cofactor(s) that mediates the interaction of Tax with its target sequence remains uncertain. Also consistent with this model for Tax function is the demonstration that a fusion protein consisting of Tax and the DNA-binding domain of GAL-4 can efficiently activate transcription through GAL-4-binding sites present in a reporter plasmid (67). Overall, these properties of Tax are therefore reminiscent of the VP16 *trans*-activator of herpes simplex virus type 1. Like Tax, VP16 is dependent on a cellular protein, in this case Oct-1, to mediate specific binding to its DNA target sequence. This dependence can again be circumvented by direct fusion of VP16 to a heterologous sequence-specific DNA-binding domain (see reference 154 for a review).

If Tax does indeed act via a cellular DNA-binding protein belonging to the CREB/ATF family, it appears possible that Tax also affects the expression of certain cellular genes containing the CREB/ATF-binding motif. In fact, transcription of the cellular proto-oncogene c-*fos* can be activated by Tax via a CREB/ATF-like sequence (65, 150). It is therefore possible that the specific interaction of one or more CREB/ATF proteins with Tax contributes to the oncogenic potential of this viral regulatory protein.

In addition to transcriptional activation through the CREB/ATF motif, Tax has also been shown to activate transcription from promoters containing the target site for the distinct cellular transcription factor NF-κB (10, 119, 179). NF-κB is normally sequestered in the cytoplasm as a inactivate complex with the cellular protein I-κB (118). Tax has been shown to induce NF-κB function by both activating the nuclear translocation of NF-κB and increasing the expression of NF-κB mRNA (5). Activation of NF-κB enhances expression of several cellular gene products including the α-chain of the interleukin-2 (IL-2) receptor (IL-2Rα) and the lymphokines IL-2 and granulocyte-macrophage colony-stimulating factor (10, 119, 143, 179, 219). The simultaneous induction of both IL-2 and IL-2Rα expression in infected T cells is believed to lead to an autocrine stimulation of T-cell replication and has therefore been proposed as a key step in the pathway leading to T-cell transformation (219, 229).

In addition to cellular genes, several viral promoter elements contain NF-κB sites and are therefore predicted to be responsive to Tax. This activation has been most clearly demonstrated with the LTR of HIV-1, which contains two binding sites for NF-κB (148, 189, 192). It has therefore been hypothesized that infection with HTLV-I might well enhance the rate of spread of HIV-1, and hence the onset of AIDS, in dually infected individuals (189).

The observation that Tax can activate promoters containing target sites for two different cellular transcription factors suggested that Tax can affect viral and/or cellular gene expression via two distinct mechanisms. Direct evidence in favor of this hypothesis has been provided by the observation that certain missense mutations of Tax retain the ability to activate the HTLV-I LTR but not the HIV-1 LTR, and vice versa (192). It therefore appears likely that Tax contains distinct functional domains responsible for specific interactions with at least two cellular factors. Similarly, several transcription factors encoded by the DNA tumor viruses, e.g., adenovirus E1A and simian virus 40 T antigen, are also known to functionally interact with multiple cellular factors that are critical to their in vivo function (154). However, the cellular targets for Tax appear to be distinct from those seen by E1A and T antigen.

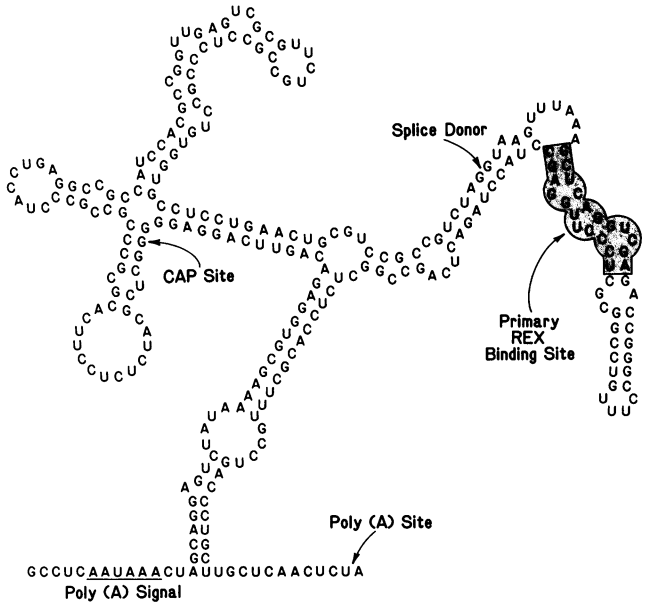

FIG. 9. Predicted RNA secondary structure of the HTLV-I RxRE. The RxRE is encoded in the HTLV-I LTR U3 and R elements. These border at the site of viral transcription initiation (CAP site). The primary binding site for Rex on the RxRE is shaded. This site lies 3′ to the major 5′ splice donor site used during viral mRNA processing. The RxRE also separates the poly(A) signal (AAUAAA) from the site of mRNA polyadenylation.

Rex Protein

The second essential regulatory protein encoded by HTLV-I, termed Rex, is functionally analagous to the Rev protein of HIV-1 (Fig. 2) (172). Both Rex and Tax are encoded by a single, multiply spliced bicistronic mRNA species (149). Rex induces the cytoplasmic expression of the unspliced and singly spliced mRNAs that encode the structural proteins of HTLV-I and inhibits the expression of this Tax/Rex-specific mRNA (Fig. 3) (81, 88, 92). The RNA target sequence for the 27-kDa Rex protein, the Rex response element (RxRE), is a complex RNA step-loop structure located in the U3 and R regions of the HTLV-I LTR (Fig. 9) (81, 212). Although RNA sequences located within R, i.e., 3′ to the cap site, are believed to be sufficient for Rex responsiveness, splicing of HTLV-I RNA would delete this 5′ RxRE (Fig. 9) (4, 21, 105). The unspliced HTLV-I genomic mRNA is therefore predicted to contain two functional copies of the RxRE, whereas *env* mRNA and the

Rex-nonresponsive mRNA that encodes Tax and Rex would each retain one RxRE sequence. In contrast, the single-copy lentivirus RRE is located within the viral envelope gene and is therefore absent from the multiply spliced, Rev-nonresponsive viral transcripts.

As expected, Rex binds the RxRE with high specificity in vitro (11, 22, 75, 213). Both mutational analysis and modification interference assays identify a short RxRE RNA sequence, encoded within the LTR R element, as the primary binding site of Rex (Fig. 9) (4, 11, 21–23). This site bears no evident similarity to the primary binding site for Rev in the HIV-1 RRE (Fig. 6). Nevertheless, it has been clearly demonstrated that Rex can rescue a Rev-deficient HIV-1 provirus and can also bind specifically to the HIV-1 RRE in vitro (4, 22, 172, 213). This apparent paradox has been resolved by the observation that the target sequence for Rex in the HIV-1 RRE is located between positions 130 and 190 in the RRE (Fig. 6), i.e., is fully distinct from the Rev primary binding site (4, 22). It is therefore likely that the ability of Rex to function through the HIV-1 RRE simply reflects the fortuitous presence of a structured sequence able to bind Rex. As predicted by this hypothesis, Rev is unable to bind to, or act through, the RxRE (4, 22, 172). Nevertheless, this apparent coincidence does underline the fact that Rex and Rev have similar mechanisms of action.

Although Rev and Rex are functionally comparable, it appears clear that these retroviral proteins have distinct domain organizations. Like Rev, Rex contains an arginine-rich motif that not only serves as a nuclear/nucleolar localization signal (191) but also is critical for binding to the RxRE (22). However, the basic motif in Rex is located at the extreme N terminus instead of near the center of the protein (191). Further, the arginine motif of Rex, although necessary, is clearly not sufficient for sequence-specific RNA binding (24, 111). Instead, additional amino acid residues in Rex, located distal to the N terminus, appear to be primarily responsible for sequence specificity (24). Although in vitro evidence indicates that Rex, like Rev, also multimerizes on its RNA target sequence (22), neither the amino acid residues involved in this process nor the physiological significance of Rex multimerization has been established.

If Rev and Rex act via the same mechanism, it is predicted that they should both interact with the same cellular cofactor(s). As described above, an essential leucine-rich motif located toward the C terminus of both HIV-1 and visna virus Rev has been proposed to serve this function. Recently, a leucine-rich motif that is functionally interchangable with this Rev activation domain has been identified in the Rex protein of HTLV-I (90, 221). In conclusion, although the domain organization of Rex is distinct from that observed in Rev, Rex nevertheless retains analogous sequence motifs that appear to subserve functions identical to those previously defined in Rev.

An interesting aspect of the HTLV-I RxRE is its role in the polyadenylation of HTLV-I transcripts. Normally, the poly(A) signal (AAUAAA) is located no more than ~30 nucleotides 5′ to the site of mRNA polyadenylation (166). The sole exception to this rule occurs in HTLV-I and the other T-cell leukemia viruses (3, 212). Here, polyadenylation occurs no less than ~250 nucleotides 3′ to the AAUAAA. This distance reflects the presence of the RxRE between the poly(A) signal and the poly(A) site (Fig. 9). It has now been demonstrated that the RxRE is tolerated because it folds to precisely juxtapose these widely separated RNA sites, thereby mimicking the normally critical short linear distance (Fig. 9) (3). This curious observation therefore provides an independent confirmation of the in vivo existence of the predicted RxRE RNA secondary structure at the 3′ terminus of HTLV-I mRNAs.

In addition to Tax and Rex, HTLV-I encodes a 21-kDa protein that results from initiation of translation at a second, internal AUG present in the Rex open reading frame (149). This protein does not display detectable Rex function (172) and has no known role in the HTLV-I replication cycle. The existence of additional HTLV-I gene products cannot be excluded at present. Of interest, HTLV-I and the related HTLV-II and bovine leukemia virus (BLV) all contain a large (~650-bp) region between the viral *env* and *tax/rex* genes that appears to be noncoding (Fig. 2). Such a coding gap would, however, appear improbable, given that retroviral genomes are normally tightly packed. Indeed, adjacent retroviral genes frequently overlap one another in different reading frames (Fig. 2). It has therefore been suggested that potential open reading frames present within this proviral sequence might well encode one or more additional HTLV-I proteins (147). Analysis of HTLV-I mRNA expression patterns by the polymerase chain reaction has indeed led to the isolation of spliced HTLV-I transcripts with the potential to encode two proteins derived from these open reading frames (147). However, it remains unclear whether either of these proteins is actually expressed in vivo. An alternative suggestion is that this intergenic region might encode an mRNA derived from the antisense strand (169). However, no direct evidence for such an mRNA species, or for the novel viral promoter required to drive its expression, has been presented.

In addition to HTLV-I, the T-cell leukemia virus subgroup includes HTLV-II, simian T-cell leukemia virus, and BLV. Current data suggest that the regulatory proteins encoded by these viruses are similar in both function and mechanism of action (21, 33, 105). However, whereas the Tax proteins of HTLV-I and HTLV-II are cross-reactive, BLV Tax appears to be only marginally functional on the HTLV-I LTR promoter element (161, 186). In contrast, HTLV-I, HTLV-II, and BLV Rex all display the ability to efficiently cross-*trans*-activate viral structural gene expression via any of the three similar RxRE sequences (57, 105).

SPUMAVIRUSES

Auxiliary Proteins of HSRV

Spumaviruses, also called foamy viruses, are a ubiquitous family of apparently nonpathogenic retroviruses (63, 89, 138). Spumaviruses have been recovered from several primate species including humans, chimpanzees, orangutans, and gorillas, as well as from a wide variety of monkeys (63, 89, 138). Indeed, HSRV was the first human retrovirus to be isolated in culture (1). In addition, spumaviruses have been recovered from cats, hamsters, cattle, and sea lions (89, 138). In culture, spumaviruses are highly cytopathic, giving rise to characteristic, multinucleated syncytia that have a vacuolated "foamy" appearance (89). Although most spumavirus isolates grow preferentially in fibroblastic cells, lymphotropic spumaviruses have been isolated from several primate species, including chimpanzees (151, 153). Animal experiments indicate that spumavirus infections have few, if any, acute pathogenic effects but do not exclude the possibility of a delayed disease syndrome, perhaps in a small

proportion of infected animals (89, 222). Interestingly, spumaviruses efficiently establish latent infections in the brain and neural tissues of animals (89), whereas transgenic mice expressing the auxiliary genes of HSRV have been shown to display clear neurological abnormalities (26). The question of whether spumaviruses have the potential to cause disease, particularly including neurological syndromes, therefore remains to be fully addressed. Although spumavirus infections appear rare in humans in North America and in Europe, HSRV has recently been shown to be endemic in certain populations in Africa (125, 222).

Although there has recently been a marked increase in scientific interest in these ubiquitous retroviruses, our knowledge of gene regulation in spumaviruses remains relatively rudimentary. However, the recent isolation of a full-length replication competent proviral clone of HSRV (121, 170) should greatly facilitate research in this area. Inspection of this proviral genome reveals two striking characteristics. The first is the unusually large size of the genome of HSRV when compared with other retroviral species (Fig. 2). The second is the existence, between *env* and the LTR, of three open reading frames that have been termed Bel-1, Bel-2, and Bel-3 (63).

An analysis of viral mRNA splicing patterns in HSRV-infected cells has revealed a level of complexity comparable to that seen with HIV-1 (146). In addition to several singly spliced mRNA species that appear to encode Env, HSRV expresses a whole set of multiply spliced mRNAs with the potential to encode the three predicted Bel proteins. Analysis of viral protein expression in HSRV-infected cells has confirmed the expression of the ~36-kDa Bel-1 and the ~42-kDa Bel-2 proteins (121). Immunofluorescence studies have shown that Bel-1 is concentrated in the nucleus, but excluded from the nucleoli, of expressing cells (Fig. 10) (102). In contrast, Bel-2 is confined to the cell cytoplasm (121). The expression of the Bel-3 open reading frame, which is not conserved in the related simian foamy virus (SFV), remains to be confirmed at the protein level.

In addition to mRNAs specific for Bel-1 and Bel-2, HSRV encodes high levels of a multiply spliced mRNA in which the N-terminal 88 amino acids of Bel-1 are spliced in frame with, but 36 amino acids in front of, the initiation codon for Bel-2 (146). The ~54-kDa protein predicted by this mRNA species, termed Bet, is expressed at high levels in HSRV-infected cells (121). Immunofluorescence analysis indicates that Bet is concentrated in the cytoplasm and specifically excluded from the cell nucleus (Fig. 10). Currently, the roles of Bel-2, Bet, and Bel-3 in the HSRV replication cycle remain unknown. It is, however, clear that *Bel-1* encodes a *trans*-activator of HSRV LTR-dependent transcription (102, 171, 216) and that Bel-1 function is critical for HSRV replication in culture (121).

The primary DNA target site for Bel-1 is located ~100 bp 5′ to the site of viral transcription initiation, within the large HSRV LTR U3 region (102, 171, 216). In addition, a second LTR sequence located ~400 bp 5′ to the cap site appears capable of mediating a partial Bel-1 response (216). However, the precise identity of the DNA target sequence(s) for Bel-1, and the question of whether Bel-1 binds the sequence directly or acts via a cellular protein, remain to be determined. The distantly related SFV has recently also been shown to encode a protein that is structurally and functionally similar to Bel-1 and that also activates viral transcription through a bipartite target sequence located in the LTR U3 element (139). However, the transcriptional *trans*-activators of HSRV and SFV are reportedly unable to cross-*trans*-activate (139).

An interesting aspect of Bel-1 is that this HSRV-specific protein can activate transcription from the LTR of HIV-1 (101, 117). This phenomenon is specific, since several other promoters, including the LTR of HTLV-I, do not respond to Bel-1. Bel-1 is also distinct from Tax in that it does not act via the HIV-1 LTR NF-κB sites. Instead, the target site for Bel-1 in the HIV-1 LTR has been mapped within a discrete ~40-bp element located immediately 5′ to these sites (101, 117). *trans*-Activation of the HIV-1 LTR by Bel-1 can readily exceed an order of magnitude and might therefore be expected to enhance the replication of HIV-1 in dually infected individuals (101). Although this possibility remains speculative, it is worth noting that the simultaneous infection of cultured human T lymphocytes by both HIV-1 and a lymphotropic chimpanzee foamy virus has been reported (151).

Although HSRV encodes a transcriptional *trans*-activator and displays the pattern of mRNA processing predicted for a complex retrovirus, it has not yet been shown to fulfill all the criteria that characterize this classification. In particular, it remains unknown whether HSRV gene regulation is temporally regulated by the action of a virally encoded equivalent of HIV-1 Rev. The cytoplasmic location of Bel-2 and Bet makes these proteins unlikely candidates for this role. However, the large HSRV genome may encode additional, currently unidentified proteins, while the function, and indeed the existence, of Bel-3 remains to be addressed. Clearly, it also remains possible that spumaviruses have developed an alternative strategy to regulate the pattern of viral mRNA expression in infected cells.

CONCLUSIONS

Complex retroviruses are distinguished from simple retroviruses not only on the basis of their larger coding capacity but, more specifically, by a particular pattern of viral gene expression that is dependent on the action of two virally encoded regulatory proteins functionally analogous to the Tat and Rev proteins of HIV-1 (Fig. 3). As I have discussed at length in this review, the various retrovirus transcriptional *trans*-activators can display highly diverse mechanisms of action. They are, however, similar to Tat in that they all increase transcription from the homologous retrovirus LTR. In contrast, current evidence suggests that proteins functionally equivalent to HIV-1 Rev, such as HTLV-I Rex, have maintained a very similar mechanism of action. Presumably, this conservation may reflect the requirement for a functional interaction with a specific, highly conserved cellular factor that is critical to the process of mRNA processing and/or transport. The observation that Rev can function in cells derived from every metazoan species tested thus far, including quail and *Drosophila* species (93, 132), may support this hypothesis.

Although complex retroviruses share a similar pattern of viral gene regulation, the in vivo consequences of this shared strategy remain unclear. It is, however, notable that all complex retroviruses appear to give rise to long-term, chronic infections marked by high levels of latently infected cells. As noted above, it has been suggested that the level of Rev activity in the infected cell may well be a primary determinant of the ability of these viruses to establish a latent infection (71, 164). It therefore seems possible that the pattern of gene regulation observed in complex retroviruses is specifically designed to allow the maintenance of retrovi-

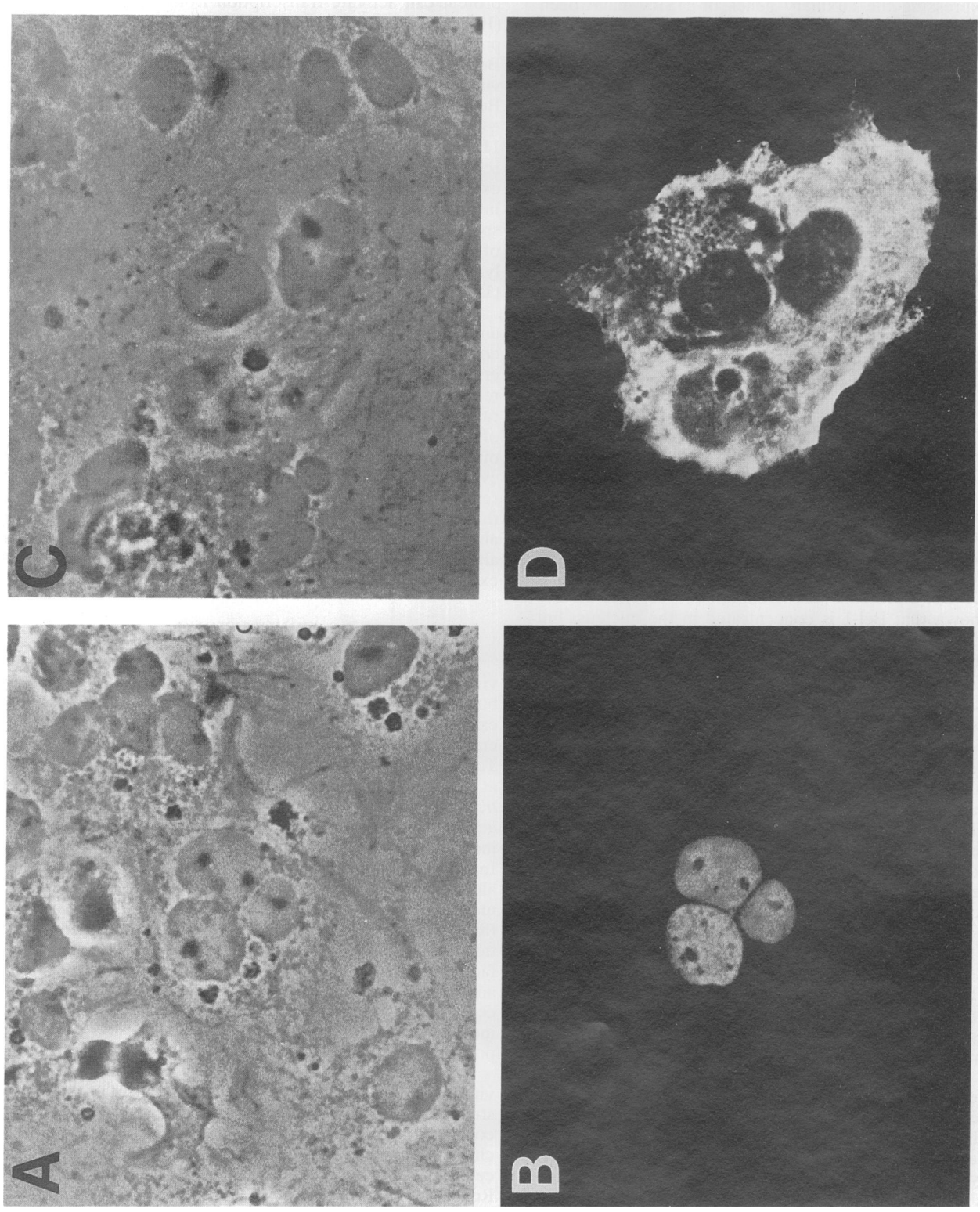

FIG. 10. Subcellular localization of the HSRV Bel-1 and Bet proteins. Phase-contrast (A and C) and immunofluorescence (B and D) photographs of cells transfected with expression vectors that encode Bel-1 (panels A and B) or Bet (panels C and D). Bel-1 is concentrated in the cell nuclei but excluded from the nucleoli. Bet is excluded from the nucleus and expressed exclusively in the cell cytoplasm. The rabbit antisera against Bel-1 and Bet used in this experiment have been described previously (121).

rus infection in the face of an ongoing host immune response.

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