A Novel Human Immunodeficiency Virus Type 1 Protein, *tev*, Shares Sequences with *tat*, *env*, and *rev* Proteins

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We have characterized a novel 28-kilodalton protein, p28^{tev}, detected in human immunodeficiency virus type 1-infected cells. *tev* is recognized by both *tat* and *rev* monospecific antibodies. *tev* is initiated at the *tat* AUG and contains the first exon of *tat* at its amino terminus, a small portion of *env* in the middle, and the second exon of *rev* at its carboxy terminus. A cDNA clone producing *tev* was cloned and expressed in human cells. Sequence analysis revealed that the *tev* mRNA is generated by splicing to a novel exon located in the *env* region. This identifies a fourth class of multiply spliced human immunodeficiency virus mRNAs, produced in infected and transfected cells. *tev* is regulated during the virus life cycle similarly to the other regulatory proteins, *tat*, *rev*, and *nef*, and displays both *tat* and *rev* activities in functional assays. Since *tev* contains important functional domains of *tat* and *rev* and is produced very early after transfection, it may be an important regulator in the initial phase of virus expression. Another *rev*-related protein, p18^{6Drev}, containing *env* and *rev* sequences, was characterized and was found not to have detectable *rev* activity.

The expression of human immunodeficiency virus type 1 (HIV-1) in human cells is regulated by viral protein factors. Two of these factors, *tat* and *rev*, are essential for viral replication (10, 13, 18, 58). *tat* interacts with a *cis*-acting element (TAR) in the R region of the long terminal repeat (LTR) (3, 33, 43, 51, 61) and increases the steady-state levels of all viral mRNAs (8, 48, 61). *rev* interacts with a *cis*-acting element in the *env* region that we have named *rev* responsive element (RRE) and affects the transport from the nucleus to the cytoplasm and the half-life of the mRNAs containing RRE (11, 12, 14, 15, 28, 29, 41, 52). A third viral protein, *nef*, has been proposed to act as a negative regulator of virus production (2, 27, 38, 45), although its function remains controversial (29a, 35). These factors are integrated into a feedback regulatory circuit that allows the balanced expression of the viral mRNAs and proteins (14, 15, 30, 60). *tat* and *rev* are encoded by overlapping reading frames and are expressed by multiply spliced mRNAs. Three different *tat* mRNAs, six *rev* mRNAs, and three *nef* mRNAs have been cloned as cDNA from infected cells (3, 42, 56a; B. K. Felber, C. M. Drysdale, and G. N. Pavlakis, manuscript submitted for publication). Low levels of a second form of a functional *tat* (1-exon *tat*) are produced from additional mRNAs (40; Felber et al., submitted).

Here we report the characterization of an additional HIV-1 protein, named p28^{tev}, which is produced by an additional mRNA(s), contains *tat*, *env*, and *rev* sequences, and is probably a fourth regulatory factor of HIV-1. Another *rev*-related protein, designated p18^{6Drev}, containing *env* and *rev* sequences was also characterized.

MATERIALS AND METHODS

Recombinant constructs. The infectious HIV-1 proviral clone HXB2 (17) was used for all constructions. Mutant fB was constructed by the insertion of 4 nucleotides (nt) at the unique *Bam*HI site at position 8021 of HXB2c (13, 28, 58). This resulted in the frameshifting of *rev* to the γ reading frame and of *env* to the *rev* reading frame (see Fig. 2A).

Mutant 107, a derivative of pHXB2gpt, has a stop codon in place of the sixth amino acid of *rev* (54). Double mutant 107fB has both the fB and 107 mutations. Double mutant 80fB has both the fB and an AUG-to-UGA mutation at the *tat* initiator codon (54). In the deletion mutant Δ BS-SAL, nt 257 to 5332 were removed from HXB2gpt by digestion with *Bss*HII and *Sal*I followed by religation of the filled-in ends, which regenerated a *Sal*I site. In this construct, *tat* is the first open reading frame. pNL1.4.7 is a *tat* cDNA clone containing exons 1, 4, and 7 (see Fig. 2A; exon numbering is by the method of Muesing et al. [42]), flanked by the HIV-1 LTRs, which provide promoter and polyadenylation signals (56a). pNL1.4.6D.7 and pNL1.5.6D.7 are the cloned p28^{tev} and p18^{6Drev} cDNAs, respectively, flanked by the HIV-1 LTRs (see below). These plasmids have the indicated introns removed precisely and express single authentic HIV mRNAs without further splicing. The numbering system used here follows the corrected HXB2 sequence (44, 50; G. N. Pavlakis, unpublished data), where +1 is the mRNA start site. The exons are numbered by the methods of Muesing et al. (42) and Schwartz et al. (56a). pL3tat produces the 1-exon *tat* protein of 72 amino acids, using the HIV-1 LTR promoter (Felber et al., submitted). pL3crev has been described as pL3ctrs (15) and produces *rev*, using the HIV-1 LTR promoter. Plasmid SL contains the HIV-1 LTR promoter and expresses a mutant *rev* protein in which Leu-81 was substituted with Phe-Pro-Arg-Gly-Glu; this protein has been shown to display a transdominant *rev* phenotype (B. Mermer, B. K. Felber, M. Campbell, and G. N. Pavlakis, Nucleic Acids Res., in press).

Transfections. Plasmid DNA was prepared by using the standard Triton X-100 clear lysis procedure and was purified twice through cesium chloride gradients. HeLa cells were transfected by using a modification (16; Felber et al., submitted) of the calcium phosphate coprecipitation technique (25). Cultures were harvested for RNA or protein analysis 1 or 2 days posttransfection as specified in the text. For analysis of transactivation, HL3T1 cells were transfected and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (61).

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Immunoprecipitations. Radiolabeling and immunoprecipitations were performed as described elsewhere (Felber et al., submitted). Briefly, cultures were labeled for 3 h with [35 S]cysteine (200 μ Ci/ml) or with 32 P_i (267 μ Ci/ml). Cultures were then rinsed and lysed in 0.5 $\times$ RIPA buffer (28). Lysates were frozen and thawed, clarified, and preadsorbed with normal rabbit serum and protein A-Sepharose. Supernatants were then incubated with the indicated sera, and the immune complexes were precipitated with protein A-Sepharose and subjected to electrophoresis in sodium dodecyl sulfate (SDS)–15% polyacrylamide gels (37). Gels were enhanced (4), dried, and exposed to film. The following rabbit antisera were used: T, raised against a synthetic peptide corresponding to the first 62 amino acids of *tat*; Rw, raised against the intact *rev* protein (provided by P. Chanda); and Rc, raised against a synthetic peptide corresponding to the 13 carboxy-terminal amino acids of the *rev* protein. The specificities of these antisera were established in control experiments. Immunoprecipitation of gel-purified proteins was carried out as follows. [35 S]cysteine-labeled proteins were eluted from minced gel fragments by incubating overnight at 4°C in 0.5 $\times$ RIPA buffer containing protease inhibitors and apomyoglobin as a carrier. Gel fragments were removed by centrifugation, and supernatants containing the individual proteins were immunoprecipitated with anti-*tat* and anti-*rev* antisera as described above.

Immunofluorescence and immunoblot analysis. Indirect immunofluorescence was performed on transfected cultures as described before (15), using rabbit anti-*rev* (Rc) or rabbit anti-*tat* (T) sera followed by fluorescein-labeled goat anti-rabbit antibody. For immunoblot analysis, proteins were subjected to SDS-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose, and detected by using a mixture of HIV-specific sera followed by 125 I-labeled protein A, all as described before (28).

RNA isolation, cDNA synthesis, and cloning. Total RNA was isolated from HXB2-infected H9 cells or mutant 107-transfected HeLa cells, using the guanidinium isothiocyanate-cesium chloride method (5). A 1- μ g portion of the RNA was reverse transcribed in the presence of a primer located in exon 7 (nt 8047 to 8066), followed by polymerase chain reaction amplification, using sense and antisense oligonucleotide primers specific for exons 1 and 7 (nt 173 to 197 and nt 8047 to 8066, respectively) or exons 4 and 7 (nt 5329 to 5351 and 8047 to 8066, respectively) (for details, see reference 56a). After phenol-chloroform extraction and ethanol precipitation, the amplified products were ligated as *Sac*I blunt-end fragments into the *Sac*I and *Eco*RV sites of plasmid Bluescript KS (a modified Bluescript plasmid). Ligated DNA was transformed into DH5 α cells (Bethesda Research Laboratories), and transformants were plated in the presence of Bluo-gal (Bethesda Research Laboratories). Plasmid DNA was prepared from white colonies and digested with restriction enzymes to determine insert size. DNA sequences were determined by the dideoxy-chain termination procedure of Sanger et al. (56), using the Sequenase version 2.0 kit (United States Biochemicals). To construct pNL1.4.6D.7 and pNL1.5.6D.7, the *tev* and p18^{6Drev} cDNAs were inserted into a cDNA expression plasmid (56a). The *Bss*HII-to-*Bam*HI fragments were ligated with the 7.1-kilobase-pair *Bss*HII-to-*Bam*HI fragment of pNL43 (1), which provided the HIV-1 LTR promoter and polyadenylation signal.

Cyanogen bromide peptide mapping. Gel fragments containing immunoprecipitated, [35 S]cysteine-labeled proteins were rehydrated and treated with cyanogen bromide at a

concentration of 70 mg/ml for 1 h as described before (46). The gel fragments were then rinsed extensively to remove the cyanogen bromide and soaked in SDS-polyacrylamide gel sample buffer, and the solubilized peptides were separated on 20% *N,N'*-diallyltartardiamide-cross-linked SDS-polyacrylamide gels.

Tryptic peptide analysis. p28^{tev}, p22, and 1-exon *tat* were immunoprecipitated from transfected cultures that had been biosynthetically labeled for 3 h with a mixture of [35 S]cysteine (200 μ Ci/ml), [35 S]methionine (200 μ Ci/ml), [3 H]leucine (167 μ Ci/ml), and [3 H]lysine (167 μ Ci/ml). The proteins were separated on an SDS-polyacrylamide gel and visualized after enhancing the gel with sodium salicylate. The protein bands were excised from the gel, rinsed to remove the fluor, and digested with trypsin; two-dimensional separations of the resulting peptides on thin-layer plates were performed as described by Gibson (23). Electrophoresis was carried out at pH 4.7 in butanol-pyridine-acetic acid-water (2:1:1:36); chromatography was carried out in butanol-pyridine-acetic acid-water (97:75:15:60). Fluorograms were prepared after spraying the plates with En³Hance (New England Nuclear).

RESULTS

Novel 28-kDa protein present in HIV-infected cells. Immunoprecipitation of proteins produced by HIV-1-infected human lymphocytes (Molt/IIIB) with a monospecific anti-*tat* antiserum revealed the presence of a protein of 28 kilodaltons (kDa) in addition to the two previously identified forms of *tat*, ~16 (2-exon *tat*) and ~14 (1-exon *tat*) kDa (Fig. 1A, lane 1). The 28-kDa protein (p28) was also observed in cultures of human lymphocytes productively infected by the molecularly cloned HIV-1 strains HXB2 and HXB3 (Fig. 1B, lanes 2 and 5). A *tat*-related protein with similar mobility has been observed previously in cells overproducing *tat* protein (13, 61). Interestingly, p28 produced from chronically infected cells (e.g., H9/HXB2) was also immunoprecipitated by an antiserum raised against the 19-kDa *rev* protein (Fig. 1B, lane 3). This suggested that p28 contained both *tat* and *rev* sequences or that it was a different protein found in a complex with the two viral regulatory factors.

Characterization of p28. The amount of p28 found in chronically infected cells was low. To characterize p28 further, we studied its production by molecular clones of HIV-1 proviruses after transfection into human cells. Initial experiments revealed that transfections of the intact provirus also resulted in low amounts of p28. Since we have shown that the intact provirus produces low levels of the regulatory factors *tat*, *rev*, and *nef* due to feedback regulation (14, 15), we examined proviral mutants deficient in *rev* in an effort to maximize expression of p28 (Fig. 2A). A frameshift mutation was introduced into the HIV-1 proviral clone HXB2 by insertion of 4 nt at the *Bam*HI site (nt 8021) (13, 28, 58), resulting in mutant fB. This mutation introduced frameshifts in both the *rev* and *env* reading frames (Fig. 2A). In a second mutant, 107 (54), the codon for the sixth amino acid of *rev* was replaced by a stop codon.

The results of immunoprecipitations of cells transfected with these proviral mutants are shown in Fig. 3. Mutant 107 produced easily detectable levels of p28 immunoprecipitated by the anti-*tat* antiserum (Fig. 3A, lane 1). p28 was also immunoprecipitated by using two different monospecific anti-*rev* antisera: Rw, generated against the intact *rev* protein (Fig. 3A, lane 4); and Rc, generated against the 13 carboxy-terminal amino acids of *rev* (Fig. 3A, lane 2). In

FIG. 1. A 28-kDa *tat*- and *rev*-related protein expressed in HIV-1-infected cells. Lysates of radiolabeled T-cell line Molt4 infected by HIV-1 (strain IIIB, panel A) or of the lymphoid cell line H9 infected by HIV-1 molecular clone HXB2 or HXB3 (panel B) were immunoprecipitated, using normal rabbit serum (P), a rabbit antiserum raised against the first 62 amino acids of *tat* (T), or a rabbit antiserum raised against the intact *rev* protein (Rw). Radiolabeling and immunoprecipitations were performed and analyzed in SDS-15% polyacrylamide gels as described in Materials and Methods.

addition to p28 and 2-exon *tat*, mutant 107 produced a protein of about 18 kDa which was recognized by both of the anti-*rev* sera but not by the anti-*tat* antiserum (Fig. 3A, lanes 2 and 4). This *rev*-related protein is described below.

Instead of p28, mutant fB produced a *tat*-related protein of 22 kDa (p22; Fig. 3B, lane 1). p22 also reacted with the antiserum generated against the intact *rev* protein (Fig. 3B, lane 3), but it did not react with the antiserum raised against the 13 carboxy-terminal amino acids of *rev* (not shown). We reasoned that the disappearance of p28 and appearance of p22 could be caused by the frameshift mutation introduced in the *Bam*HI site of HIV-1. To prove this hypothesis, we introduced a frameshift mutation at the *Bam*HI site of mutant 107, resulting in the double mutant 107fB. Cells transfected with this mutant produced a 22-kDa protein instead of p28 (Fig. 3C, lane 1). These results demonstrated that p28 contained amino acids encoded by a reading frame spanning the *Bam*HI site. Since p28 was immunoprecipitated by the anti-*rev* antisera (Fig. 1B and 3A), we concluded that this reading frame was the second exon of *rev*. After addition of 4 nt at the *Bam*HI site, the *rev* coding region shifted to a smaller coding region (labeled γ in Fig. 2A). As a result, 60 carboxy-terminal amino acids of the *rev* coding region were replaced with 20 amino acids of the γ coding region. This caused a net loss of 40 amino acids from p28, resulting in a

protein of ~22 kDa, which is in agreement with our observations. Since the frameshift mutation at the *Bam*HI site resulted in the loss of the common carboxy terminus of *rev* and p28, the anti-*rev* antibody directed against the carboxy terminus of *rev* did not recognize p22 from cells transfected by the mutant fB, as mentioned above. These results indicated that p28 and p22 contain *tat* sequences and some *rev* sequences encoded upstream of the *Bam*HI site and that p28 also contains the carboxy-terminal part of *rev*.

To rule out the possibility that p28 was coprecipitated as a complex with *tat* and *rev*, p28 was eluted from an SDS-polyacrylamide gel fragment and reprecipitated with the anti-*tat* and anti-*rev* antisera. Both antisera recognized gel-purified p28 (Fig. 3D, lanes 1 and 3). This established that p28 does in fact share epitopes with *tat* and *rev* and is not precipitated as a result of protein complex formation or nonspecific binding. We have named the protein *tev* (p28^{tev}) to indicate its relation to both *tat* and *rev*.

***tev* starts at the *tat* AUG.** To define the genomic region encoding p28^{tev} in more detail, a series of HXB2-derived mutants were analyzed for the ability to produce the protein. Figure 4A shows results obtained with deletion mutant Δ BS-SAL. This mutant, constructed by deleting the *gag*, *pol*, and *vif* regions of the proviral clone HXB2, contains *tat* as the first open reading frame (Fig. 2A). Δ BS-SAL produced easily detectable levels of p28^{tev} and 2-exon *tat* and lower levels of 1-exon *tat* after transfection into HeLa cells (Fig. 4A, lane 4). Only p28^{tev}, but no *rev* protein, was detected by using anti-*rev* antiserum (lane 5). Since the *tat* AUG is the first AUG in this construct, this result suggested that the *tat* initiation codon is used for the production of p28^{tev}.

To verify that *tev* initiates at the *tat* AUG, we tested the ability of a derivative of proviral mutant 80*tat*⁻, which contains a stop codon in place of the *tat* AUG (AUG to UAG) (54), to produce *tev*. This mutant is *rev*⁺ and, as expected, produces low levels of regulatory factors upon cotransfection with a *tat* expression plasmid. To overcome this, we introduced a frameshift mutation at the *Bam*HI site of 80*tat*⁻, generating the double-mutant 80fB (*tat*⁻ *rev*⁻). 80fB did not produce p22 (i.e., the expected frameshifted form of p28^{tev}) when cotransfected with a *tat* expression plasmid into HeLa cells (data not shown). On the basis of these findings, we concluded that p28^{tev} is a novel protein of HIV-1 that initiates at the *tat* AUG and contains *tat* sequences at its amino terminus and *rev* sequences at its carboxy terminus.

***tev* is a phosphoprotein.** The *rev* protein is known to be phosphorylated (7, 31); in contrast, *tat* is not phosphorylated, although it contains many serines and threonines. To determine whether either p28^{tev} or p22 bears this posttranslational modification, HeLa cells transfected with fB, mutant 107, or a mixture of pL3*tat* and pL3*crev* were labeled with ³²P_i and analyzed by immunoprecipitation, using the anti-*tat* antiserum; [³⁵S]cysteine-labeled cultures were analyzed in parallel (data not shown). Only p28^{tev} was phosphorylated (Fig. 4B, lane 1), while p22 was not (data not shown). This suggested that p28^{tev} contains a portion of the *rev* domain bearing this modification and that this domain lies 3' to the *Bam*HI site, since it is absent from p22. Estimation of the ³²P and ³⁵S incorporated into p28^{tev} or *rev* demonstrated that phosphorylation of p28^{tev} was low compared with that of *rev* (data not shown).

Expression of *tev* is regulated by *rev*. It has been proposed that HIV-1 undergoes two different phases of gene expression (14, 15, 30, 60). The early phase is characterized by high

FIG. 2. Schematic representation of recombinant plasmids (A) and structure and amino acid sequence of p28^{rev} (B). (A) The open reading frames for *tat*, *rev*, *env*, and *y* of the HIV-1 clone HXB2c are indicated (not in scale). The genomic region necessary for the expression of p28^{rev} is indicated at the top. *rev* mutants *fB* and 107, deletion mutant Δ BS-SAL, and cDNA expression plasmids pNL1.4.7 (encoding *tat*), pNL1.4.6D.7 (encoding *rev*), and pNL1.5.6D.7 (encoding p18^{rev}) are described in Materials and Methods. The positions of the splice sites are indicated. Bs, *Bss*HII; S, *Sal*I. (B) Arrows indicate the boundaries of exons 4 to 6D and 6D to 7. Amino acid sequence analysis of the 38 residues encoded by exon 6D revealed the presence of four potential N-glycosylation sites (i.e., Asn-X-Ser/Thr), two methionines, and two cysteines.

levels of regulatory mRNAs and proteins (*tat*, *rev*, and *nef*), while the late phase is characterized by high levels of structural protein production and low levels of regulatory factors. Direct measurements of viral mRNA and protein production have demonstrated that feedback regulation by *rev* protein exerted at the mRNA level is necessary for the switching from early to late phase (15; Felber et al., submitted). Analysis of infection of lymphocytes by HIV has also suggested that the multiply spliced species appear earlier (34).

To study the effect of *rev* on p28^{rev} production, we transfected Δ BS-SAL into HeLa cells in the presence or absence of the *rev* expression plasmid pL3crev. As men-

tioned above, mutant Δ BS-SAL produced easily detectable levels of p28^{rev} and 2-exon *tat* and lower levels of 1-exon *tat* (Fig. 4A, lane 4). The presence of *rev* protein produced by pL3crev in the same cell resulted in decreased expression of p28^{rev} and 2-exon *tat* and increased expression of 1-exon *tat* (Fig. 4A, cf. lanes 1 and 4). Thus, p28^{rev}, like 2-exon *tat*, is down regulated in the presence of *rev*. This finding indicates that p28^{rev} belongs to the group of regulatory factors (*tat*, *rev*, and *nef*) produced early in the virus life cycle from multiply spliced mRNAs lacking the RRE sequence and are indirectly down regulated by *rev* (15; Felber et al., submitted).

Structural analysis of *tev*. To define the structure of *tev*

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1      /      1
ATG GAG CCA GTA GAT CCT AGA CTA GAG CCC TGG AAG CAT CCA GGA AGT CAG CCT AAA ACT
met glu pro val asp pro arg leu glu pro trp lys his pro gly ser gln pro lys thr
61      /      21
GCT TGT ACC AAT TGC TAT TGT AAA AAG TGT TGC TTT CAT TGC CAA GTT TGT TTC ATA ACA
ala cys thr asn cys tyr cys lys lys cys cys phe his cys gln val cys phe ile thr
121     /      41
AAA Gcc tta gGc atc tcc tat ggc agG AAG AAG CGG AGA CAG CGA CGA AGA GCT CAT CAG
lys ala leu gly ile ser tyr gly arg lys lys arg arg gln arg arg arg ala his gln
181     /      61
AAC AGT CAG ACT CAT CAA GCT TCT CTA TCA AAG CAT TTA AAG TGC ACT GAT TTG AAG AAT
asn ser gln thr his gln ala ser leu ser lys his leu lys cys thr asp leu lys asn
241     /      81
GAT ACT AAT ACC AAT AGT AGT AGC GGG AGA ATG ATA ATG GAG AAA GGA GAG ATA AAA AAC
asp thr asn thr asn ser ser ser gly arg met ile met glu lys gly glu ile lys asn
301     /      101
TGC TCT TTC AAT ATC AGC ACA AGC ATA AGA GAC CCA CCT CCC AAT CCC GAG GGG ACC CGA
cys ser phe asn ile ser thr ser ile arg asp pro pro pro asn pro glu gly thr arg
361     /      121
CAG GCC CGA AGG AAT AGA AGA AGA AGG TGG AGA GAG AGA CAG AGA CAG ATC CAT TCG ATT
gln ala arg arg asn arg arg arg arg trp arg glu arg gln arg gln ile his ser ile
421     /      141
AGT GAA CGG ATC CTT GGC ACT TAT CTG GGA CGA TCT GCG GAG CCT GTG CCT CTT CAG CTA
ser glu arg ile leu gly thr tyr leu gly arg ser ala glu pro val pro leu gln leu
481     /      161
CCA CCG CTT GAG AGA CTT ACT CTT GAT TGT AAC GAG GAT TGT GGA ACT TCT GGG ACG CAG
pro pro leu glu arg leu thr leu asp cys asn glu asp cys gly thr ser gly thr gln
541     /      181
GGG GTG GGA AGC CCT CAA ATA TTG GTG GAA TCT CCT ACA GTA TTG GAG TCA GGA ACT AAA
gly val gly ser pro gln ile leu val glu ser pro thr val leu glu ser gly thr lys
601     /      201
GAA TAG
glu AMB

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FIG. 2—Continued.

further, [³⁵S]cysteine-labeled p28^{tev} and p22 proteins were treated with cyanogen bromide to cleave at methionines, and the resulting peptides were analyzed in SDS-polyacrylamide gels (Fig. 5A). Cleavage of p28^{tev} (lane 2) resulted in two fragments with apparent sizes similar to those of 1-exon *tat* (lane 3) and *rev* (lane 1) proteins. Cleavage of p22 (lane 5) resulted in a fragment with the approximate mobility of 1-exon *tat* and a smaller fragment apparently corresponding to the shorter carboxy-terminal portion of p22. Since no internal methionines are present within *tat* or *rev*, we reasoned that p28^{tev} contains additional sequences between the *tat* and *rev* portions, including at least one methionine.

Additional structural information on p28^{tev} was derived from two-dimensional peptide analysis of 1-exon *tat*, p28^{tev}, and p22 proteins radiolabeled with [³⁵S]cysteine, [³⁵S]methionine, [³H]leucine, and [³H]lysine (Fig. 5B). As expected, the patterns obtained for p28^{tev} and p22 were more complex than those of 1-exon *tat*. All three peptides in the 1-exon *tat* pattern were also present in p28^{tev} and p22, including an intensely labeled peptide likely to correspond to a portion of the cysteine-rich domain. Five additional peptides were common to p28^{tev} and p22 but absent from 1-exon *tat*; one of these peptides was intensely labeled. *rev* contains only two cysteine residues, both in the carboxy-terminal region, which is absent from p22 due to the frameshift mutation. Since the intensely labeled peptide was observed in both p28^{tev} and p22, it is more likely to represent a fragment of the methionine-containing domain suggested by the cyanogen bromide patterns (Fig. 5A) than a *rev* peptide.

The p28^{tev} pattern also contained peptides not present in p22 (e.g., peptide marked A); these peptides may represent the portion of the *rev* protein encoded from the sequence 3' to the *Bam*HI site which is absent in p22.

***tev* is expressed by a novel mRNA species.** Two mechanisms of *tev* production were considered: p28^{tev} could be produced by an additional mRNA that splices the first part of *tat* to the second part of the *rev* open reading frame in such a way that a shift from the *tat* to the *rev* frame is produced. Alternatively, *tev* could be produced from the *tat* mRNA by ribosomal frameshifting from the *tat* to the *rev* reading frame. To examine the latter possibility, the proteins produced from two *tat* cDNA expression clones (pNL1.3.4.7 and pNL1.4.7; see references 42 and 56a), one of which is shown in Fig. 2A, were examined. These expression plasmids produce authentic *tat* mRNAs after transfection in HeLa cells. Only the expected 2-exon *tat*, but not p28^{tev}, was produced in these experiments (56a). This result suggested that p28^{tev} is produced by an additional mRNA(s).

To identify new mRNA species, RNA was extracted from HXB2-transfected or -infected cells. cDNAs were synthesized and amplified by using the polymerase chain reaction technique. Different sets of primers located in exons 1 and 7 or exons 4 and 7 were used as detailed elsewhere (56a; also see Materials and Methods). The amplified products were analyzed on polyacrylamide gels and cloned in a modified Bluescript vector. The cDNA libraries prepared from RNAs isolated from transfected or infected cells were screened for clones containing exons 4 and 7. Both libraries yielded a

FIG. 3. Evidence that the 28-kDa protein is related to *tat* and *rev*. (A, B, C) HeLa cells were transfected with the indicated plasmids, labeled with [35 S]cysteine, and subjected to immunoprecipitation, all as described in Materials and Methods. The following rabbit sera were used: P, normal rabbit serum; T, anti-*tat* serum raised against the first 62 amino acids of *tat*; Rc, anti-*rev* serum raised against the 13 carboxy-terminal amino acids of *rev*; Rw, anti-*rev* serum raised against the intact *rev* protein. Lanes marked M contain 14 C-labeled Amersham Rainbow protein molecular weight standards. The ratio of p28 or p22 to *tat* was higher when the cells were labeled 1 day after transfection (panel C) compared with 2 days after transfection (panels A and B). (D) Immunoprecipitation of gel-purified p28 and *tat* proteins. [35 S] cysteine-labeled p28 and *tat* proteins were eluted from polyacrylamide gel fragments, and the individual proteins were immunoprecipitated, using anti-*tat* (lanes 1, 2) or anti-*rev* (lane 3) antiserum as described in Materials and Methods.

cDNA species containing an additional novel exon as identified by sequencing. This exon, designated 6D (Fig. 2A), contains 116 base pairs and is generated by utilization of a novel splice acceptor (nt 6153) and a novel splice donor (nt 6270). This cDNA clone has an open reading frame that starts at the *tat* AUG, continues in the *env* reading frame within exon 6D, and is followed by the second exon of *rev* (Fig. 2B). In agreement with the prediction made from the cyanogen bromide cleavage patterns (Fig. 5A), exon 6D contains two methionine residues. Both methionines are contained in one of several potential tryptic fragments. It is likely that this methionine-containing peptide corresponds to the intensely labeled tryptic peptide shared by p22 and p28^{rev} (Fig. 5B).

To verify that the 1.4.6D.7 cDNA encodes p28^{rev}, the cloned cDNA was inserted between the HIV-1 5' and 3' LTRs in a plasmid designed for expression in eucaryotic cells. The 1.4.6D.7 expression plasmid was transfected into HeLa cells, and gene products were radiolabeled and detected by immunoprecipitation. A single protein species corresponding to p28^{rev} was detected by using anti-*tat* and anti-*rev* antisera (Fig. 6). Only p28^{rev} was detected by using HIV-positive human serum (data not shown); *tat*, *rev*, and *nef* proteins were not detected with monospecific rabbit

antisera (Fig. 6; anti-*nef* data not shown). These results established that *rev* is the only protein expressed from the 1.4.6D.7 cDNA.

Southern blot analysis of a library of exon 6D-containing cDNAs that had been prepared from RNAs from HIV-1-infected cells by using primer pairs in exons 1 and 6D revealed that, in addition to exon 4, exon 6D can be spliced to exon 4A, 4B, or 5 (56a). These results were confirmed by cloning and sequencing each cDNA. cDNAs 1.4A.6D.7 and 1.4B.6D.7 contain an open reading frame that begins with the *rev* initiation codon and terminates in exon 6D, yielding a peptide of 37 amino acids. In addition, these cDNAs, as well as cDNA 1.5.6D.7, contain a second open reading frame that initiates at either of the two closely spaced internal methionines in exon 6D (Fig. 2B) and ends with the second exon of *rev*. cDNA 1.5.6D.7 was transferred into a eucaryotic expression plasmid and transfected into HL*tat* cells, and the produced proteins were detected by immunoprecipitation. A protein of about 18 kDa was detected with anti-*rev* serum; this protein comigrated with the 18-kDa *rev*-related species detected in mutant 107-transfected cells (Fig. 3A; data not shown). We concluded that this protein, designated p18^{6Drev}, corresponds to the species predicted to initiate at one of the two closely spaced methionines in exon 6D and to

FIG. 4. Localization of the genomic region encoding $p28^{tev}$ (A), which is a phosphoprotein (B). Cells were transfected with deletion mutant $\Delta BS-SAL$, with (lanes 1 to 3) or without (lanes 4 to 6) *rev* expression plasmid L3crev (15), labeled 1 day later with [^{35}S] cysteine, and analyzed by immunoprecipitation, using anti-*tat* (T) or anti-*rev* (Rw) antiserum or normal rabbit serum (P). (B) Cells were transfected with mutant 107 (lane 1) or with the *tat*-producing plasmid pL3*tat* plus the *rev*-producing plasmid pL3crev (lanes 2 and 3). Two days later, the cells were labeled with [^{35}S]cysteine or $^{32}P_i$ as described in Materials and Methods and analyzed by immunoprecipitation, using anti-*tat* (T), anti-*rev* (Rc), or normal rabbit serum (P). Shown here is a composite of the resulting gel.

terminate with the second exon of *rev*. Results of anti-*nef* immunoprecipitations showed that the 1.5.6D.7 cDNA also expressed significant amounts of *nef* protein (i.e., it is bicistronic [data not shown]).

$p28^{tev}$ is localized in the nucleoli of transfected cells. *tat* and *rev* have been shown to accumulate primarily in the nucleoli of transfected cells (7, 9, 14, 15, 33, 47, 53). This targeting has been attributed to the presence of stretches of basic amino acids located between residues 48 and 56 of *tat* and between residues 35 and 50 of *rev*; both of these signals are present in $p28^{tev}$. Indirect immunofluorescence images of HeLa cells transfected with the 1.4.6D.7 expression plasmid revealed that $p28^{tev}$ accumulated in the nucleoli of transfected cells (Fig. 7A), in a pattern identical to that observed for *rev* and *tat* proteins produced after cotransfection of expression plasmids pL3crev and pL3*tat* (Fig. 7B and C, respectively). In contrast, $p18^{6Drev}$, which also contains the nuclear localization signal of *rev*, was detected in both the cytoplasm and nucleoli of HL*tat* cells transfected with the 1.5.6D.7 expression plasmid (Fig. 7D).

$p28^{tev}$ displays *tat* and *rev* activities. In the experiments described above, expression of $p28^{tev}$ from the 1.4.6D.7 expression plasmid did not require coexpression of *tat* protein. This showed that $p28^{tev}$ functions as *tat*. To compare the transactivation activities of $p28^{tev}$ and *tat*, either the 1.4.6D.7 expression plasmid or a *tat* expression plasmid was transfected into cell line HL3T1, which contains stably integrated silent copies of the HIV-1 LTR ligated to the CAT gene (61). Quantitation of CAT assays performed on the cell extracts revealed a 260-fold activation by $p28^{tev}$ compared with a 370-fold activation by *tat*; thus, the transactivation activity of $p28^{tev}$ is similar to that of *tat* (data not shown).

To assess $p28^{tev}$ for *rev* activity, complementation assays were performed with the HeLa cell line HL*fb*, which contains stably integrated copies of the *rev* mutant provirus *fb* (Mermer et al., in press). These cells produce low levels of *env* glycoprotein and *gag* precursor $p55^{gag}$ and do not produce processed $p24^{gag}$ or virus particles (Fig. 8, lane 2). Lane 8 of this immunoblot shows that complementation of the *fb* mutation upon expression of *rev* in *trans* results in increased expression of *env* and *gag* which is easily assessed by the detection of $p24^{gag}$. Transfection of the *tev* cDNA expression plasmid also resulted in increased expression of *env* and accumulation of $p24^{gag}$ (lane 3) and in formation of mature virus particles (results of electron microscopic examination not shown). Thus $p28^{tev}$ displayed *rev* activity; however, its ability to complement the *fb* mutation was not as strong as that seen for authentic *rev* (cf. $p24^{gag}$ levels in lanes 3 and 8). $p18^{6Drev}$ did not display detectable *rev* activity in transfections done with the 1.5.6D.7 expression plasmid (data not shown).

Figure 8 also shows the effect of coexpression of a transdominant *rev* mutant (Mermer et al., in press) on the ability of *rev* and *tev* to complement the *fb* mutation. Complementation by *rev* was effectively inhibited at a plasmid ratio of >5:1 (mutant *rev*/authentic *rev*; Fig. 8, lanes 9 to 12); in contrast, *tev* was effectively inhibited at a ratio of 1:1 (mutant *rev*/*tev*; lanes 4 to 7). These observations suggest that the interaction of $p28^{tev}$ with RRE is comparatively weak and more readily inhibited than that of *rev*.

***tev* is an early viral protein.** To study the regulation of $p28^{tev}$ production over time, HeLa cells were transfected with mutant 107 or HXB2 (Fig. 9A and B, respectively). The cells were radiolabeled for 3 h at 12, 20, and 42 h posttransfection, and the proteins were immunoprecipitated by using the anti-*tat* antiserum. High levels of $p28^{tev}$ could be detected as early as 12 h after transfection by mutant 107. Interestingly, more $p28^{tev}$ than *tat* was produced at early times. Low levels of $p28^{tev}$ and trace amounts of 1-exon and 2-exon *tat* were also detected 12 h posttransfection by HXB2 (Fig. 9B); however, the ratio of $p28^{tev}$ /*tat* was lower than that observed for mutant 107. The presence of 1-exon *tat* at 12 h posttransfection by HXB2 suggested that *rev* was expressed at this time point. Immunoprecipitations with the anti-*rev* antisera confirmed these observations (data not shown). These data provide further evidence that $p28^{tev}$ is down regulated in the presence of *rev*. They also indicate that production of *rev* and progression of the "late" phase of viral expression occurred by 12 h posttransfection.

DISCUSSION

We have identified and characterized proteins of 28 and 18 kDa that are expressed in HIV-1-infected and -transfected cells. The 28-kDa protein, designated $p28^{tev}$, was initially

FIG. 5. Peptide analysis of p28^{rev} and p22. (A) Cyanogen bromide cleavage of p28^{rev} and p22 proteins. Gel fragments containing immunoprecipitated, [³⁵S]cysteine-labeled proteins were treated with cyanogen bromide to cleave at methionines, and the resulting peptides were separated in 20% *N,N'*-diallyltartardiamide-cross-linked polyacrylamide gels as described elsewhere (46) and in Materials and Methods. 1-Exon *tat*, 2-exon *tat*, and *rev* do not contain internal methionines and therefore were not cleaved. (B) Tryptic peptide analysis. Immunoprecipitated p28^{rev}, p22, and 1-exon *tat*, biosynthetically labeled with a mixture of [³⁵S]cysteine, [³⁵S]methionine, [³H]leucine, and [³H]lysine, were digested with trypsin, and two-dimensional separations of the resulting peptides on thin-layer plates were performed as described elsewhere (23) and in Materials and Methods. Electrophoresis was from left to right; chromatography was from bottom to top. Closed arrowheads indicate peptides common to all three proteins; open arrowheads indicate peptides shared by p28^{rev} and p22. Arrow marked A indicates a peptide found only in p28^{rev}. Parallel analysis of *rev* protein repeatedly yielded overlapping patterns of spots, precluding their interpretation.

FIG. 6. $p28^{tev}$ is expressed from pNL1.4.6D.7. HeLa cells were transfected with pNL1.4.6D.7, labeled 1 day later with [35 S]cysteine, harvested, and analyzed by immunoprecipitation, using normal rabbit serum (P) or anti-*tat* (T) or anti-*rev* (Rw) antiserum. Lane marked M contains 14 C-labeled Amersham Rainbow protein molecular weight standards. The p28 protein indicated here comigrated with $p28^{tev}$ produced by mutant 107 (data not shown).

recognized on the basis of its immunological cross-reactivity with monospecific anti-*tat* and anti-*rev* antisera. Peptide comparisons suggested that, in addition to *tat* and *rev* domains, $p28^{tev}$ contained a unique methionine-containing peptide. This was confirmed by cDNA cloning, which established that $p28^{tev}$ is a hybrid protein expressed by a novel, multiply spliced mRNA. This mRNA has a continuous open reading frame that begins with the first exon of *tat*, continues through a new exon within *env*, and ends with the second exon of *rev*. The 18-kDa protein ($p18^{Drev}$) was initiated from one of two methionines present within the *env* exon and terminated with the second *rev* exon.

The *env* domain present in $p28^{tev}$ spans amino acids 129 to 166 of gp120. This sequence includes two cysteines, two methionines, and four potential N-glycosylation sites. Results of mutation analyses indicate that this portion of gp120 is one of the domains essential for association of gp120 with gp41 (36). Based on comparisons of the immunological properties of short synthetic gp120 peptides, Goudsmit and co-workers (24) identified a portion of this domain (i.e., amino acids 135 to 148) as an immunoreactive segment of the glycoprotein. The amino-terminal *env* domain of $p18^{Drev}$ begins with Met-147 or Met-149 and ends with amino acid 166. This sequence includes one cysteine and two potential N-glycosylation sites. Although the *env* domain did not appear to affect targeting of $p28^{tev}$ to the nucleoli (Fig. 7A), it might play a role in $p18^{Drev}$'s altered targeting (Fig. 7D) and lack of *rev* activity. In this regard, it will be of interest to determine whether the *env* glycosylation sites of $p28^{tev}$ and $p18^{Drev}$ are utilized.

The entire first exon of *tat* constitutes the amino-terminal part of $p28^{tev}$. Studies of *tat* have established that the first 58 of the 72 amino acids encoded in the first exon are sufficient for full transactivation activity (8, 43, 57, 59, 61). Thus, it was not surprising that $p28^{tev}$ was nearly equivalent to *tat* in ability to transactivate the HIV-1 LTR promoter. The entire second exon of *rev* constitutes the carboxy-terminal part of $p28^{tev}$ and $p18^{Drev}$. This portion of *rev* includes the following domains: (i) a cluster of basic amino acids (residues 35 to 50), proposed to function as a nuclear localization signal; (ii) a series of highly conserved proline residues (residues 70, 72, 76, and 77); (iii) a conserved acidic domain (residues 84, 87, and 88); and (iv) a cluster of serines near the carboxy terminus, recently shown to be possible phosphate acceptors (6, 7, 9, 15, 31, 39, 47; Mermer et al., in press). The observation that $p18^{Drev}$ accumulated in both the nucleoli and cytoplasm and did not have detectable *rev* activity is in agreement with a report by Malim et al. (39) indicating that sequences in both the first and second exons of *rev* are necessary for the protein's nuclear targeting and functional activity. On the basis of these findings, one might have expected $p28^{tev}$ to exhibit some cytoplasmic targeting and to be functionally *rev*⁻. However, this proved not to be the case, since $p28^{tev}$ was localized almost exclusively to the nucleoli (Fig. 7A) and was *rev*⁺ (Fig. 8). One possible explanation for the ability of $p28^{tev}$ to function as *rev* is that sequences contributed by the protein's *tat* domain were able to substitute for the missing *rev* sequences.

$p28^{tev}$ was weakly phosphorylated compared with *rev* (Fig. 4B); in addition, $p18^{Drev}$ was also found to be very weakly phosphorylated (data not shown). Taken together, these observations suggest that sequences in the first *rev* exon may contribute to phosphorylation of the potential phosphate acceptors located near the carboxy terminus. These phosphate acceptors have been shown to be dispensable for *rev* function (6).

Mutant 107 expressed high levels of both $p28^{tev}$ and $p18^{Drev}$ (Fig. 3A). Given the finding that $p28^{tev}$ displayed *rev* activity, one might expect this mutant to display a *rev*⁺ phenotype. Results of immunoprecipitation and electron microscopy studies verified that mutant 107 is functionally *rev*⁺; i.e., it produces structural proteins and mature virus particles (data not shown). These observations highlight the importance of taking into consideration the existence and functional activity of alternative factors such as $p28^{tev}$ when constructing such point mutants of the regulatory proteins.

The functional role of $p28^{tev}$ in infected cells is not yet clear. The finding that expression of $p28^{tev}$ was decreased in the presence of *rev* (Fig. 4A) shows that $p28^{tev}$ belongs to the group of regulatory factors (*tat*, *rev*, and *nef*) that are produced early in the virus life cycle from mRNAs lacking the RRE sequence and are indirectly down regulated by *rev* (15, 17). $p28^{tev}$ is produced very early after transfection and is more abundant than *tat* at early time points (i.e., as early as 12 h posttransfection [Fig. 9]). These observations indicate that $p28^{tev}$ may play an important regulatory role in the initial phase of virus expression.

Our results show that molecular clones of HIV-1 are capable of expressing more differentially spliced mRNAs in infected cells than previously recognized. A comparison of DNA sequences of HIV-1 proviruses showed that exon 6D is conserved in the IIIB family of HIV-1 molecular clones. Other proviral clones examined lacked one or both of the exon 6D splice sites, but contained other possible alternative splice sites in the vicinity of the exon 6D sequence. This raises the possibility that proviral clones lacking exact

FIG. 7. Subcellular localization of p28^{rev} and p18^{Drev}. HeLa cells were transfected with the *rev* cDNA expression plasmid pNL1.4.6D.7 (A) or with a mixture of the *tat* and *rev* expression plasmids pL3tat and pL3crev (B and C) and analyzed by indirect immunofluorescence as described before (15). In panel D, the *tat*-expressing cell line HLtat (56a) was transfected with the p18^{Drev} cDNA expression plasmid pNL1.5.6D.7. Panel A shows p28^{rev} visualized with anti-*tat* (T); panel B shows *rev* visualized with anti-*rev* (Rc); panel C shows *tat* visualized with anti-*tat* (T); and panel D shows p18^{Drev} visualized with anti-*rev* (Rc).

FIG. 7-Continued.

matches for the exon 6D splice acceptor and donor could generate *tev*-like proteins through recognition of other alternative splice sites in *env*.

The complex expression strategy of HIV may have developed as a result of the acquisition of regulatory factors such as *rev* that ensure the production of structural mRNAs despite the presence of a multitude of splice sites. In

addition to the exon 6D-containing messages described above, we have evidence that HXB2-infected cells produce other multiply spliced mRNAs generated by utilization of other splice acceptors located between exons 4 and 7 (56a). Some of these mRNAs have the potential to express alternative forms of regulatory proteins that may have a role in HIV infection. The presence and activity of these factors

FIG. 8. $p28^{rev}$ displays *rev* activity. HLFb cells were transfected with 2 μ g of pNL1.4.6D.7 (lanes 3 to 7) or pL3crev (lanes 8 to 12), plus 0, 2, 5, or 10 μ g of a plasmid expressing a transdominant mutant of *rev* (mrev LSL; Mermer et al., in press); all transfection mixtures were adjusted to 17 μ g of DNA with salmon sperm DNA. Complementation of the *rev* mutation was analyzed by immunoblot assay as described previously (28). Lane 1 contains a lysate of HXB2-infected cells; lane 2 shows a control transfection done with 17 μ g of salmon sperm DNA. Radioactivity in the $p24^{agg}$ bands was quantitated by using the AMBIS radioanalytic imaging system (Automated Microbiology Systems, Inc.). Relative plasmid sizes were taken into consideration when calculating the mutant *rev*/*rev- Δ rev* ratios stated in the text.

FIG. 9. $p28^{rev}$ is synthesized early after transfection. HeLa cells were transfected by mutant 107 (A) or HXB2 (B) and labeled with [35 S] cysteine at 12, 20, and 42 h posttransfection. Cells were harvested and the proteins were immunoprecipitated with normal rabbit serum (P) or anti-*tat* antiserum (T) as described in Materials and Methods. The long exposure shown in panel B was required to visualize the lower amounts of *tat* and $p28^{rev}$ produced by HXB2.

must be considered for a detailed understanding of viral expression.

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