Cloning and Functional Analysis of Multiply Spliced mRNA Species of Human Immunodeficiency Virus Type 1

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We have used the polymerase chain reaction technique to clone the small multiply spliced mRNA species produced after infection of human cells by a molecular clone of human immunodeficiency virus type 1 (HIV-1). We identified six Rev-expressing mRNAs, which were generated by the use of two splice acceptors located immediately upstream of the *rev* AUG. The class of small mRNAs included 12 mRNAs expressing Tat, Rev, and Nef. In addition, HIV-1 produced other multiply spliced mRNAs that used alternative splice sites identified by cloning and sequencing. All of these mRNAs were found in the cytoplasm and should be able to produce additional proteins. The coding capacity of the *tat*, *rev*, and *nef* mRNAs was analyzed by transfection of the cloned cDNAs into human cells. The *tat* mRNAs produced high levels of Tat, but very low levels of Rev and Nef. All the *rev* mRNAs expressed high levels of both Rev and Nef and were essential for the production of sufficient amounts of Rev. Therefore, HIV-1 uses both alternatively spliced and bicistronic mRNAs for the production of Tat, Rev, and Nef proteins.

Human immunodeficiency virus type 1 (HIV-1) has the general structural and functional characteristics of lentiviruses (6, 22, 23, 36, 51, 63, 65). Lentiviruses cause slow, debilitating diseases in humans and animals. In many cases, there is a striking inability of the immune system to provide effective immunity, despite a measurable host immune response. The long latency period, the chronic course of the disease, and the failure of the immune response may be related to the expression strategy and life cycle of these viruses. Virus expression is determined to a great extent by regulatory factors encoded by short open reading frames in the viral genome. Therefore, study of the regulatory factors and their expression is important for understanding the viral life cycle and pathogenesis. For HIV-1, the most extensively studied lentivirus, two small regulatory proteins, Tat and Rev, have been shown to be essential for replication (12, 17, 21, 61). Tat is a potent transactivator of the long terminal repeat (LTR) promoter that increases the steady-state levels of all HIV-1 mRNAs (5, 10, 18, 31, 43, 46a, 48, 52, 62, 66). Transactivation of the HIV-1 LTR has been shown to be a stable property among various clinical isolates of HIV-1 with different biological characteristics (41, 58). The second essential positive activator, Rev, acts through a unique *cis*-acting element, termed the *rev*-responsive element (RRE), which is located in the *env* region (13, 16, 28, 29, 39, 53). Rev promotes the transport of RRE-containing mRNAs out of the nucleus (16, 19, 29, 39; B. K. Felber, C. M. Drysdale, and G. N. Pavlakis, submitted for publication). This increases the ratio of unspliced to spliced HIV-1 mRNAs in the infected cell, resulting in high production of viral structural proteins (17–19, 28). A third protein, Nef, has been proposed to downregulate virus replication and HIV-1 LTR-directed transcription (2, 26, 37, 46), but its function remains controversial (30, 33). Regulatory proteins similar in function to those of HIV-1 have been found in other lentiviruses, such as HIV-2 (3, 15, 27), simian immunodeficiency virus

(SIV) (9, 38, 64), visna virus (11, 24, 32, 40), and equine infectious anemia virus (14, 60).

HIV-1 mRNAs expressing Tat and Nef have been described (4, 42), but no Rev-specific mRNA had been identified, and the exact mRNA encoding Rev was not known. In this study we have identified six novel Rev-expressing HIV-1 mRNAs by cloning cDNAs amplified by the polymerase chain reaction (PCR). Extensive analysis of a cDNA library showed that the HIV-1 molecular clone HXB2 produces many different small mRNAs, including three Tat-encoding mRNAs, six Rev-encoding mRNAs, and three Nef-encoding mRNAs. Functional studies revealed that the *rev* mRNAs also produce Nef protein efficiently, while the *tat* mRNAs produce primarily Tat. These experiments indicated that the level of expression of the individual proteins from the small mRNAs depends on the initiator AUG and can be explained by the scanning model for initiation of translation (34, 35).

In addition to the Tat-, Rev-, and Nef-expressing mRNAs, many multiply spliced species containing a small exon in the *env* region were detected. These mRNAs would be expected to express alternative forms of regulatory factors, in addition to the recently described Tev protein (7). We have also identified two new splice acceptors located upstream of the last exon of *tat* and *rev* that would generate mRNAs producing novel forms of Tat or Rev.

MATERIALS AND METHODS

RNA extraction. Total RNA was extracted from HXB2-infected or -transfected cells by the guanidine thiocyanate procedure (8), and cytoplasmic RNA was prepared as described before (19).

Synthetic oligonucleotide primers. Seven amplification primers were used in this work. NARS and BSS are located in exon 1 of the HIV-1 genome (sense strand), SACS is located in exon 5 (sense strand), 3016S spans the splice donor at nucleotide (nt) 5591 (sense strand), 3423A is located in exon 6D (antisense strand), 3015A is located before the splice acceptor of exon 7 (antisense strand), and BAMA is

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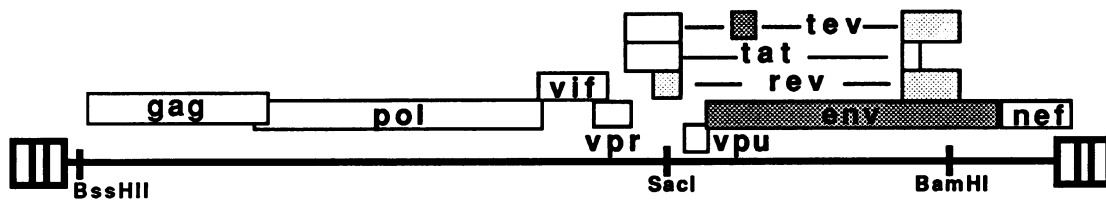
A. HIV-1 GENOME

B. EXONS
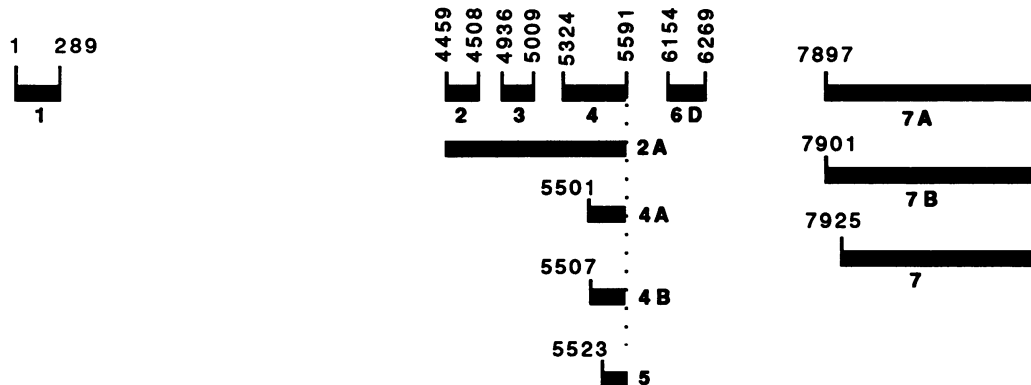
C. PCR PRIMERS

FIG. 1. (A) Organization of the HIV-1 genome. The viral open reading frames and the locations of the restriction sites used for cDNA cloning are indicated. (B) The different exons are represented by solid bars and numbered according to Muesing et al. (42) with modifications. The newly described exons 4A, 4B, 6D, 7A, and 7B are also indicated. Numbers indicate the exact positions of the exons in the HIV-1 (clone HXB2) genome. (C) The positions of the amplification primers used in this work are indicated here and detailed in Materials and Methods.

located in exon 7 (antisense strand). The locations of the amplification primers are indicated in Fig. 1. The sequences and the exact positions of the primers in the HXB2 genome are as follows: NARS, 5'-CTCTAGCAGTGGCGCCCGAA CAGGG-3' (nt 173 to 197); BSS, 5'-GGCTTGCTGAAGCG CGCACGGCAAGAGG-3' (nt 246 to 273); SACS, 5'-GAAG AAGCGGAGACAGCGACGAAGAGCTC-3' (nt 5523 to 5551); 3016S, 5'-TCATCAAGCTTCTCTATCAAAGCAGT-3' (nt 5568 to 5593); 3423A, 5'-CATCATCGCCCTCTTACT ATTACCTC-3' (nt 6195 to 6220); 3015A, 5'-CTGAAACG ATAATGGTGAATATCC-3' (nt 7901 to 7924); and BAMA, 5'-GCCAAGGATCCGTTCACTAATCGAATGG-3' (nt 8004 to 8031). The primer pair BSS-BAMA was used for amplification of all small multiply spliced mRNAs, primer pairs 3016S-BAMA and SACS-3015A were used to amplify cDNAs containing the exon 4 to 7 splice junction, and primer pair NARS-3423A was used to amplify cDNAs containing exon 6D.

Oligonucleotides used as probes were located in exon 2

(EXON2S), the intron between exons 2 and 3 (IN), exon 3 (EXON3S), exon 4 (2606S), exon 4A/4B (CO12), exon 5 (SACS) (see above), and exon 6D (3311). The sequences and the positions of these oligonucleotides are as follows: EXON2S, 5'-GGACCAGCAAAGCTCCTCTG-3' (nt 4484 to 4503); IN, 5'-CACATCCCACTAGGGGATGC-3' (nt 4752 to 4771); EXON3S, 5'-GGACACATAGTTAGCCCTAGG-3' (nt 4962 to 4982); 2606S, 5'-GGTGTCGACATAGCAGAA TAGGC-3' (nt 5329 to 5351); CO12, 5'-CTTAGGCATCTC CTATGGCA-3' (nt 5502 to 5521); and 3311, 5'-TTCACGTG ACTAAACTTCTTACTATGATTATGG-3' (nt 6158 to 6190). The numbering system follows the corrected HXB2 sequence (44, 49, 50, 51; Pavlakis, unpublished), where +1 is the mRNA start site.

cDNA synthesis and PCR amplification. Total RNA (0.2 to 1 μg) was reverse transcribed in the presence of the antisense oligonucleotide primer, and the resulting DNA was amplified in the same tube as follows. The RNA was heated at 65°C for 5 min and cooled on ice. First-strand synthesis of

cDNA was carried out in a total volume of 100 μl containing 50 mM KCl, 10 mM Tris hydrochloride (Tris-HCl, pH 8.3), 5 mM MgCl$_2$, 0.01% (wt/vol) gelatin, 200 mM each dNTP, 8 U of RNasin (Promega), 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals), and 0.5 μg of antisense oligonucleotide primer. The reaction mixture was incubated at 45°C for 2 h. PCR amplification (56) of the single-stranded DNA was carried out after addition of 0.5 μg of sense oligonucleotide primer and 0.7 U of *Thermus aquaticus* DNA polymerase (55) (Taq polymerase; Perkin-Elmer Cetus). The reaction mixture was overlaid with 2 drops of mineral oil (Sigma Chemical Co.) and incubated at 94, 55, and 72°C for 1, 2, and 3 min, respectively. This cycle was repeated 35 times in a DNA thermal cycler (Perkin-Elmer Cetus).

Analysis of PCR products. Ten-microliter portions of each PCR amplification were analyzed on 5% nondenaturing polyacrylamide gels. DNA was visualized by UV fluorescence after staining with ethidium bromide. To denature DNA before blotting, gels were soaked in 0.5 M NaOH–1.5 M NaCl for 10 min, washed twice with H$_2$O, and neutralized in 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl for 10 min. The DNA was transferred to nylon membranes by electroblotting at 35 mA for 16 h in 2× TBE buffer (0.1 M Tris-HCl [pH 8.3], 0.1 M boric acid, 2.0 mM EDTA) at 4°C. After cross-linking of DNA, the filters were prehybridized for 4 h in 4× SET (0.6 M NaCl, 0.12 M Tris-HCl [pH 8.0], 4 mM EDTA) containing 0.2% sodium dodecyl sulfate (SDS), 5 mg of salmon sperm DNA per ml, and 10× Denhardt solution (2 mg of Ficoll per ml, 2 mg of polyvinylpyrrolidone per ml, 2 mg of bovine serum albumin per ml). Hybridization was performed overnight in the same solution plus 10% dextran sulfate. The filters were washed in 1× SET containing 0.2% SDS and autoradiographed without intensifying screens.

Cloning and sequencing of amplified cDNA. After amplification of the cDNAs by PCR, the reaction mixture was extracted with phenol-chloroform and precipitated twice with ethanol in the presence of 2 M ammonium acetate (pH 8.0). The amplification products were redissolved and digested with either *Bss*HII alone or *Bss*HII and *Sac*I. cDNAs digested with *Bss*HII were cloned into a modified Bluescript vector [pBluescript KS(−); Stratagene] digested with *Bss*HII and *Sma*I. cDNAs digested with *Bss*HII and *Sac*I were cloned into the same Bluescript vector digested with *Bss*HII and *Sac*I. The positions of the restriction sites used for cloning are indicated in Fig. 1. The modified vector contained a *Bss*HII linker inserted between the unique *Hind*III and *Eco*RI sites in the vector polylinker. PCR-amplified cDNAs covering the exon 4 to 7 splice junction were digested with *Sac*I and cloned into the *Sac*I and *Eco*RV sites of the Bluescript KS(−) vector. *Escherichia coli* DH5α cells (Bethesda Research Laboratories) were transformed with the ligated DNA, and the resulting colonies were replica plated on nitrocellulose filters. The filters were hybridized to different ³²P-, 5′-end-labeled oligonucleotide probes. The hybridization temperature varied between 40 and 65°C, depending on the T_m of the oligonucleotide probe, and was carried out as described above, except that prehybridization was for 1 h and hybridization was for 2 h.

For sequencing, double-stranded plasmid DNA was denatured in 0.2 M NaOH for 5 min at room temperature, neutralized by adding 0.1 volume of 3 M sodium acetate (pH 5.5), and ethanol precipitated. The pellet was redissolved in Sequenase reaction buffer (United States Biochemical Corp.) together with the appropriate sequencing primer. The DNA sequences were determined by the dideoxy chain termination procedure of Sanger et al. (57) with the Sequenase version 2.0 kit (United States Biochemical) according to the supplier's recommendations.

Eucaryotic expression plasmids. Eucaryotic expression plasmids were constructed by replacing the *Bss*HII-*Bam*HI fragment of plasmid pNL43 (1), which contains an intact provirus inserted into plasmid pUC18, with the *Bss*HII-*Bam*HI fragments of the various cDNA clones. This provided the cDNAs with the HIV-1 LTR promoter and polyadenylation signal. After transfections in HeLa cells, these plasmids expressed individual multiply spliced HIV-1 mRNAs which were identical to the ones produced by the intact provirus. This allowed the analysis of expression of individual mRNA species. The *rev*-expressing plasmid pL3crev has been described as pL3ctrs (19).

Cell and transfections. Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. The HeLa-derived cell line HLtat contains the *tat*-expressing plasmid pL3tat, which has the HIV-1 LTR promoter linked to the first exon of *tat* followed by the simian virus 40 polyadenylation site (Felber et al., submitted). These cells constitutively express a truncated 14-kilodalton (kDa) functional form of Tat (one-exon Tat), which can be distinguished from the 16-kDa two-exon Tat expressed from the complete *tat* gene in the *tat* cDNAs. The HeLa-derived cell line HLfB (B. Mermer, B. K. Felber, M. Campbell, and G. N. Pavlakis, Nucleic Acids Res., in press) contains stably integrated copies of the *rev* mutant fB (28). Plasmid DNAs (5 μg) were transfected into different cell lines by the calcium phosphate coprecipitation technique (25) as described before (20). Medium was changed at day 1 after transfection, and cells were harvested at day 2 and analyzed for protein or RNA production.

Labeling and immunoprecipitation. HLtat cells transfected with the cDNA clones were metabolically labeled with [³⁵S] cysteine for 3 h. Cells were lysed in 0.5× RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Immunoprecipitations were carried out with rabbit preimmune (P), anti-Tat (T), anti-Rev (R), and anti-Nef (N) antisera and analyzed on 15% denaturing polyacrylamide gels as described before (Felber et al., submitted).

Western immunoblots. The transfected cells were lysed in 0.5× RIPA buffer 2 days after transfection. Proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose filters. The filters were probed with HIV-1 patient sera followed by ¹²⁵I-labeled protein A as described before (28).

RESULTS

cDNA synthesis and amplification. To investigate the structure of the small multiply spliced HIV-1 mRNAs, we amplified cDNAs from HIV-1-infected or -transfected cells by PCR. The locations of the PCR primers used to amplify cDNAs corresponding to the small mRNAs are indicated in Fig. 1C. First, cDNA was synthesized by using reverse transcriptase and the primer BAMA, located in exon 7 at the 3′ part of the provirus. (The numbering system of exons used herein follows that of Muesing et al. [42] with modifications.) The synthesized cDNAs were subsequently PCR-amplified in the same tube with the same primer in combination with primer BSS, located in exon 1 (Fig. 1). Primers in exons 1 and 7 were selected, since these exons are presumably present in all the viral transcripts. In order to distinguish cDNAs differing in size by only a few nucleotides, the
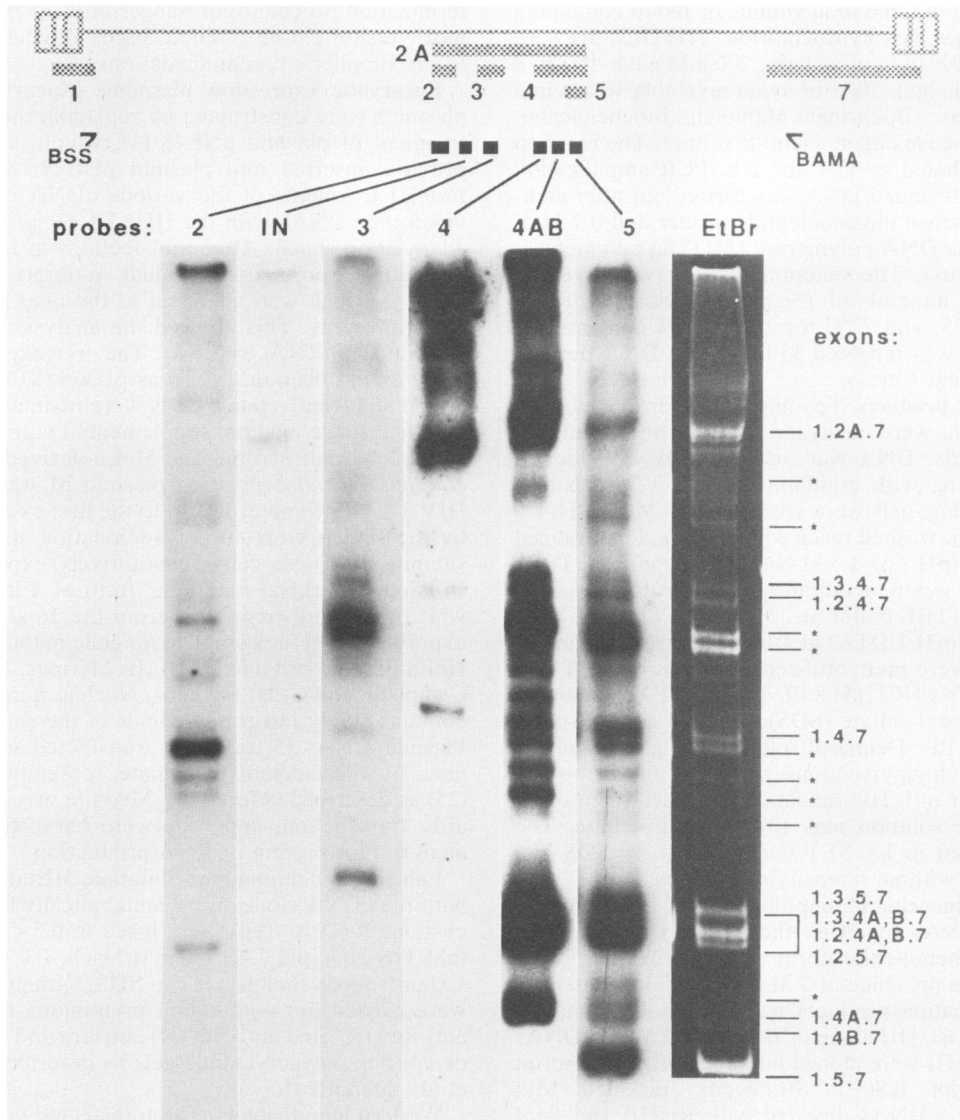
FIG. 2. Southern blots of cDNA PCR-amplified with primer pair BSS-BAMA. The positions of the amplification primers BSS, BAMA, and different previously known exons (identified by numbers) are indicated at the top. Solid bars indicate the location of the oligonucleotide probes used for hybridization. The hybridization probes used were: 2, oligonucleotide EXON2S; 3, oligonucleotide EXON3S; 4, oligonucleotide 2606S; 4AB, oligonucleotide CO12; 5, oligonucleotide SACS. For the exact positions of the oligonucleotide probes, see Materials and Methods. The assignment of the amplified bands to different mRNAs is shown on the right (see also Fig. 3A). Stars indicate bands corresponding to alternatively spliced mRNAs discussed in the text, containing exon 6D, 7A, or 7B. EtBr, Ethidium bromide.

amplification products were separated on 5% polyacrylamide gels and visualized by staining with ethidium bromide (Fig. 2, rightmost lane). A complex and reproducible pattern of bands was observed. A comparison of amplification products with RNA from HXB2-infected H9 cells or from HXB2-transfected HeLa cells resulted in the same pattern of amplified bands. Results of Southern blot analysis (described below) confirmed that these bands represented HIV-1-derived cDNAs.

Southern blot analysis. To verify that these amplified products represented HIV cDNAs and to identify the genomic regions they contained, the amplified cDNAs were transferred to nylon membranes and hybridized to different probes located in exons 2, 3, 4, or 5 and to the intron between exons 2 and 3 (Fig. 2). These exons have been shown to be present in various small HIV-1 mRNAs (4, 42).

All bands visualized by ethidium bromide staining hybridized to the exon 1 and exon 7 probes (data not shown), as well as to an exon 5 probe (Fig. 2), indicating that this region in exon 5 is present in all small multiply spliced HIV-1 mRNAs. Amplification products detected by hybridization to two different probes within exon 4 are also shown. Probe 4 hybridized to a region upstream of the *tat* AUG and probe 4AB hybridized to a region spanning the *rev* AUG. Interestingly, probe 4AB hybridized to many more bands than probe 4, indicating the existence of at least one splice site located after the *tat* AUG but before the *rev* AUG. Hybridization with probes in exons 2 and 3 showed that these small, noncoding exons were present in many cDNAs. We also used a probe located between exons 2 and 3, named IN. This probe should hybridize to an mRNA containing exon 2A (Fig. 1), which may form mRNAs expressing Vif protein (4).

A.

— tev —
— tat —
— rev —

gag   pol   vif   vpr   vpu   nef

EXONS: 2   3   4   6D   7A
        1   4A   7B
                4B   7
                5

mRNAs:

3 tat mRNAs

tat 4   nef tev
tat   nef tev
tat   nef tev

6 rev mRNAs

4A/4B   nef tev
         nef tev
         nef tev

3 nef mRNAs

5   nef
      nef
      nef

B. OTHER mRNAs

tev

4   tat -----   tev nef
             AUG
4A/4B   tev nef
             AUG
5   tev nef

tat   7A   tev nef
tat   7B   tev

2A   vif   tat   vpr   tev nef
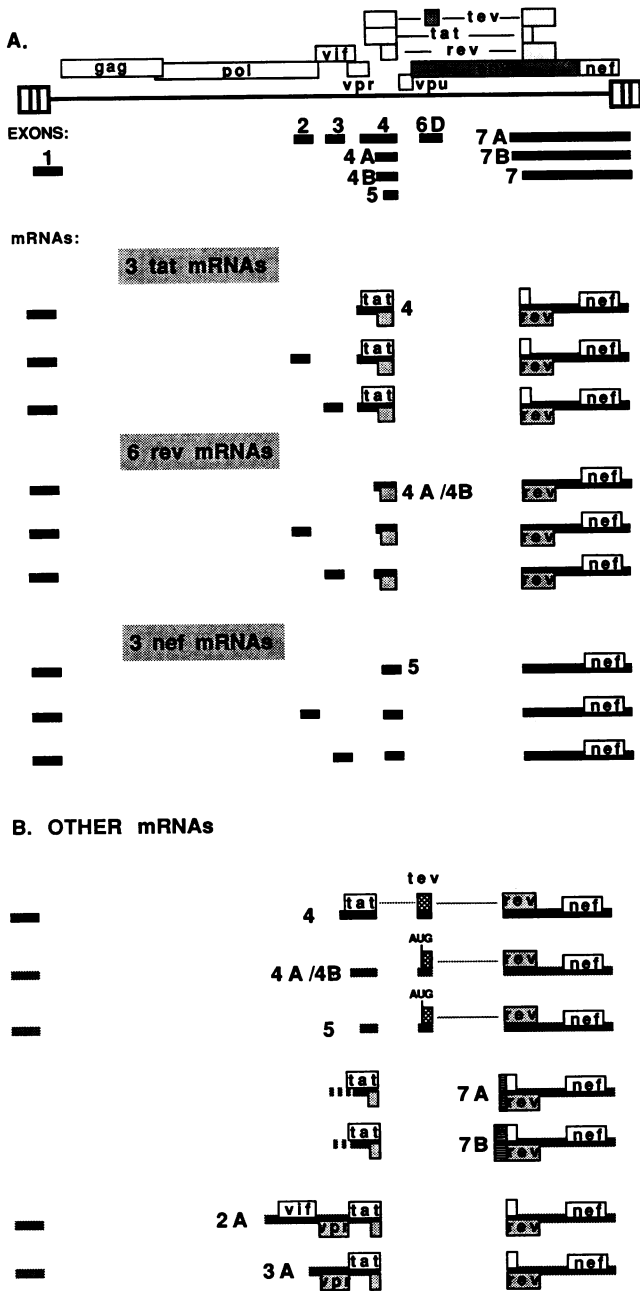3A   tat   vpr   tev nef

FIG. 3. Small multiply spliced HIV-1 mRNAs. (A) Genomic organization of HIV-1 and the different exons are shown at the top. The structures of the multiply spliced mRNAs encoding Tat, Rev, and Nef identified by cloning and sequencing are shown. These mRNAs fall into three groups, characterized by their middle exon, 4, 4A/4B, or 5. Tat is encoded by a group of three mRNAs, containing exon 4. Rev is encoded by two groups of mRNA, containing either exon 4A or 4B. The Nef protein is encoded by three mRNAs containing exon 5. Each group consists of three mRNA species characterized by the absence or presence of the two small noncoding exons 2 and 3. (B) Structures of additional HIV-1 multiply spliced mRNAs. Exon 6D-containing cDNA 1.4.6D.7 has been cloned and sequenced (7; unpublished). The other species were shown to exist by the PCR amplification and hybridization experiments. Multiply spliced mRNAs containing the alternative splice acceptors 7A and 7B, upstream of the previously known exon 7, are also indicated. Such mRNAs may produce variant forms of regulatory factors Tat and Rev containing additional amino acids, indi-

Only one band hybridized to this probe, indicating that only one exon 2A-containing mRNA was generated. The structure of this mRNA is shown in Fig. 3B. Although we were able to assign several amplified bands to specific cDNAs based on their sizes and hybridization to different probes, many bands could not be assigned by using these criteria. To investigate the exact nature of the PCR-amplified molecules, we analyzed the amplification products by cloning and sequencing.

Cloning and sequencing of cDNAs. The PCR-amplified cDNAs were cloned into a modified Bluescript vector and sequenced by a double-stranded DNA sequencing protocol as described in Materials and Methods. As expected from the results of the hybridization experiments, mRNAs generated by utilization of additional splice sites between the *tat* and *rev* AUGs were identified. We cloned two different types of cDNAs having *rev* as the first open reading frame. These mRNAs contained either of the two small exons, named 4A and 4B, utilizing splice acceptors located 15 and 9 nt upstream of the *rev* AUG, respectively (Fig. 3; see also Fig. 7). These two exons, whose exact locations are shown in Fig. 1, were found spliced to exon 1 either directly or via exon 2 or exon 3, generating six different *rev* mRNAs (Fig. 3). These findings verify the results of S1 nuclease analysis that identified exons 4A and 4B in RNA from HIV-1-infected or -transfected cells (Felber et al., submitted). Others have also reported the presence of one splice site in the same region between the *rev* and *tat* AUGs by S1 nuclease analysis (54). This proposed splice acceptor corresponds to 4B, identified here by cDNA cloning and sequencing.

We also cloned and sequenced cDNAs corresponding to the previously described *tat* mRNAs containing exon 4 (4, 42). These three variant mRNAs are designated here as 1.4.7, 1.2.4.7, and 1.3.4.7, according to the exons they contain (Fig. 3). Sequence analysis of clones containing exon 5 confirmed and extended previous studies, which had identified two exon 5-containing mRNAs, 1.5.7 and 1.2.5.7 (4, 42). In line with observations made for the *tat* and *rev* mRNAs, the group of mRNAs containing exon 5 was also found to consist of three mRNAs (Fig. 3), generated by splicing of exon 5 to exon 1 directly or via exon 2 or exon 3. Therefore, all four central overlapping exons of the virus, 4, 4A, 4B, and 5, existed in three combinations: spliced to exon 1 directly or linked to exon 1 via either exon 2 or 3, yielding a total of 12 different mRNAs.

The 12 *tat*, *rev*, and *nef* mRNA species identified by cloning and sequencing did not account for the number of cDNA bands amplified by PCR (Fig. 2), suggesting the presence of additional mRNAs. Since total RNA had been used in the initial amplifications, it could be argued that not all of the observed cDNAs represented cytoplasmic mRNAs. To determine whether this was the case, both cytoplasmic RNA and total RNA were used for amplification, and patterns of bands were analyzed in parallel. The complex profile obtained with total RNA was essentially identical to that of amplified cytoplasmic RNA. The possibility of a small amount of nuclear contamination in the cytoplasmic fraction could not be ruled out; however, if this had occurred, we would have expected a lower amount of amplification products in the cytoplasmic fraction. These

cated by striped boxes. Two additional multiply spliced mRNAs predicted to exist in infected cells and designated 1.2A.7 and 1.3A.7 are probably expressing Vif and Vpr, respectively. Grey bars indicate exons not entirely cloned.
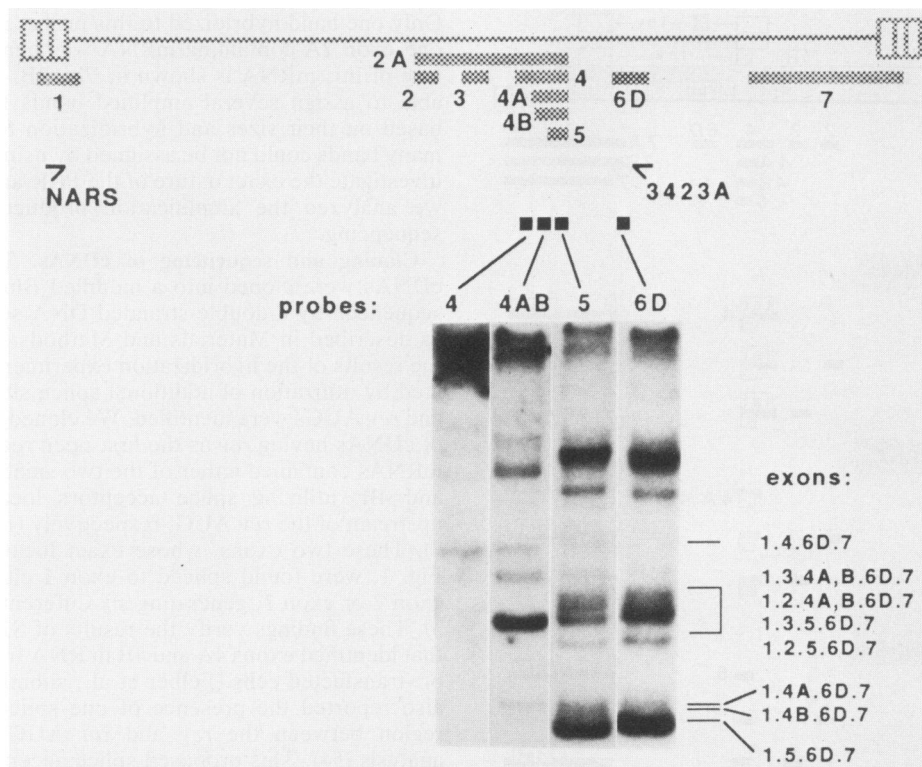
FIG. 4. Southern blots of cDNA PCR-amplified with primer pair NARS and 3423A. The positions of the amplification primers NARS and 3423A and different previously known exons are indicated at the top. Numbers indicate different exons. Solid bars indicate the location of the oligonucleotide probes used for hybridization. The hybridization probes used were: 4, oligonucleotide 2606S; 4AB, oligonucleotide CO12; 5, oligonucleotide SACS; 6D, oligonucleotide 3311. Several bands hybridizing to probe 6D also hybridized to probes located in exons 4, 4A/4B, and 5, indicating that exon 6D can be spliced to all four middle exons (Fig. 3B), resulting in additional mRNAs. The assignment of some bands is shown on the right.

results suggested that the unidentified cDNA bands represent cytoplasmic mRNAs.

Identification of additional HIV splice sites. To obtain more information about the additional multiply spliced mRNAs, we focused our attention on the region between exons 4 and 7. We have recently identified another small exon, named exon 6D, located within the *env* gene, as indicated in Fig. 3. We have shown that one mRNA containing exons 1, 4, 6D, and 7 generates a hybrid protein, Tev (7), consisting of the first exon of Tat, 38 amino acids of Env, and the second exon of Rev. We reasoned that this exon might be spliced not only to exon 4 but also to exons, 5, 4A, and 4B. This could generate a maximum of 12 additional mRNAs, differing from the mRNAs depicted in Fig. 3 only by the presence of exon 6D spliced to exon 7. To test this hypothesis, we synthesized a primer located in exon 6D (primer 3423A, Fig. 1C). This primer was used for cDNA synthesis of RNA isolated from HXB2-infected or -transfected cells and subsequent PCR amplification in combination with the NARS primer located in exon 1. The cDNAs were subjected to Southern blot analysis with probes located in exon 4, 4A/4B, or 5 (Fig. 4). These experiments demonstrated that exon 6D was spliced to exon 4, 4A, 4B, or 5, indicating that at least 4 and possibly 12 mRNAs containing exon 6D exist in HXB2-infected cells. The similar results obtained in amplifications with BSS-BAMA and NARS-3423A indicated that mRNAs containing the small exons 2 and 3 in addition to 6D were also produced. The presence of exon 6D-containing mRNAs can partially account for the additional unassigned bands detected in Fig. 2. The structures and coding potential of four of these

mRNAs (1.4.6D.7, 1.4A.6D.7, 1.4B.6D.7, and 1.5.6D.7) are shown in Fig. 3B. It has been shown that HIV-1-infected cells produce Tev from 1.4.6D.7 mRNA (7) and that a protein containing 20 amino acids of Env linked to the second exon of Rev can be produced from 1.4A.6D.7, 1.4B.6D.7, or 1.5.6D.7 mRNAs (7).

To identify any additional splice sites between the splice donor utilized by exons 4, 4A, 4B, and 5 and the splice acceptor of exon 7 (nt 5591 to 7925, Fig. 1), we PCR-amplified mRNAs containing this region with two primers, 3016S and 3015A, designed to detect alternative splice sites. Primer 3016S extends 4 nt 3′ of the splice donor at nt 5591 (Fig. 1), and primer 3015A extends 25 nt 5′ of the exon 7 splice acceptor at nt 7925, thereby selectively amplifying either unspliced or alternatively spliced mRNAs in this region. cDNA synthesis and PCR amplification with primer pair 3016S and BAMA did not result in amplification products corresponding to alternatively spliced mRNAs, indicating that only the previously described splice donor at nt 5591 (Fig. 1B) was utilized in this region. Sequence analysis of cDNAs amplified with primers SACS and 3015A revealed that this splice donor could be spliced to two additional splice acceptors, located upstream of the exon 7 splice acceptor (nt 7925). The corresponding exons were designated 7A and 7B (Fig. 1 and 7), with splice acceptors located 28 and 24 nt upstream of exon 7, respectively. Splicing of these exons to exon 4 would produce mRNAs with the capacity to express a Tat protein with an insertion of eight amino acids between the two coding exons or a Tat-Env hybrid protein, respectively. If these exons could be spliced

to exons 4A and 4B, the mRNAs produced would express either a Rev protein with eight additional amino acids or a Rev-Tat hybrid protein. The presence and significance of such proteins in infected cells remains to be investigated.

In order to identify other alternatively spliced small mRNAs, we replica-plated several hundred colonies from the cDNA library and screened them with probes 2, 3, 4, 4AB, and 5. Using this approach, we did not identify any additional cDNAs. We were also unable to find any mRNA species containing both exons 2 and 3, indicating that exons 2 and 3 are mutually exclusive within the multiply spliced mRNAs. In addition, no cDNAs containing exons 1, 2, or 3 linked directly to exon 7 were found. Results of colony hybridizations with oligonucleotide 3015A as a probe revealed that exon 7A and 7B splice acceptors were present in less than 1% of the cDNA clones, which indicates that these splice acceptors are rarely used by the virus.

Protein production by the cDNA clones. The coding potential of the various cDNAs was examined by gene transfer experiments. In order to express individual cDNAs in HeLa cells, they were transferred into eucaryotic expression vectors that provided the HIV-1 LTR promoter and polyadenylation site (see Materials and Methods). This strategy resulted in eucaryotic expression vectors producing authentic HIV-1 mRNAs without missing or extraneous nucleotides. Figure 5A shows the results obtained after transfection of one cDNA expression plasmid from each group (i.e., pNL1.5.7, pNL1.4A.7, or pNL1.4.7; see Fig. 3) into HLtat cells, a HeLa cell line constitutively expressing the first exon of the HIV-1 Tat protein (Felber et al., submitted). Protein products were biosynthetically labeled and immunoprecipitated with anti-Tat (T), anti-Rev (R), or anti-Nef (N) antisera (Fig. 5A). These experiments showed that pNL1.5.7 produced Nef, while pNL1.4A.7 produced high levels of both Rev and Nef. The expression of Rev and Nef from plasmid pNL1.4B.7 was similar to that from pNL1.4A.7 (data not shown). pNL1.4.7 produced high levels of the 16-kDa two-exon Tat protein. Expression of low levels of Rev and Nef from pNL1.4.7 could only be detected in very long exposures of these gels. To exclude the possibility that the expressed mRNAs were further spliced after transfections, RNA from cells transfected with the various cDNA expression plasmids was isolated and PCR-amplified as described above with primers BSS and BAMA. No evidence of splicing was detected in these experiments (data not shown), verifying that the same mRNA molecule produced more than one protein.

The effect of the presence of the small exons 2 and 3 on the expression of cDNAs was examined by comparing the levels of Nef protein expressed by the three *nef* cDNAs, which differ only in the presence or absence of exons 2 or 3 (Fig. 5B). Comparisons of Nef protein production after immunoprecipitations with anti-Nef (N) antiserum demonstrated that the three different cDNA clones were expressed equally well. Similar results were obtained with the different cDNAs expressing Rev and Tat (data not shown). From these results we conclude that the small noncoding exons 2 and 3 do not play a significant role in the expression of the multiply spliced mRNAs.

The levels of Tat and Rev proteins produced by the different cDNAs were also examined by functional assays. Activation of the HIV-1 LTR by Tat was tested after transfections of the different cDNAs into HL3T1 cells. These cells contain stably integrated copies of the HIV-1 LTR linked to the chloramphenicol acetyltransferase (CAT) gene (47, 66). The *tat* cDNAs were able to activate CAT
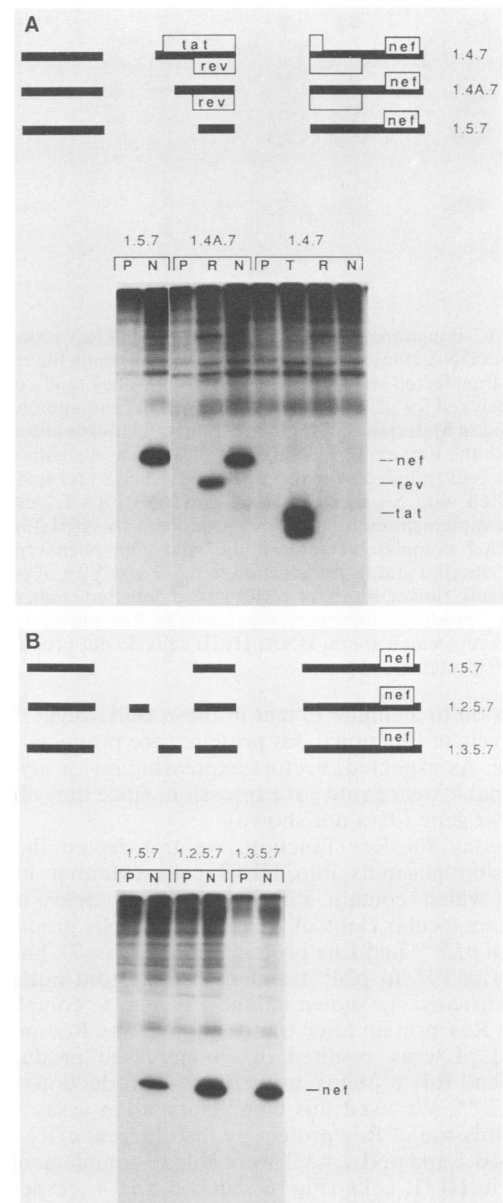

FIG. 5. Immunoprecipitations of Tat, Rev, and Nef expressed from cDNA clones. (A) HLtat cells transfected with the cDNA expression plasmids pNL1.5.7, pNL1.4A.7, and pNL1.4.7 (structures are shown at the top) were biosynthetically labeled, and the cell lysates were immunoprecipitated with preimmune serum (P) and anti-Tat (T), anti-Rev (R), and anti-Nef (N) antisera. pNL1.4.7 produced high levels of Tat but only long exposures of the gels revealed the presence of low levels of Rev and Nef. Two proteins were precipitated by the anti-Tat serum; the upper band corresponds to the complete two-exon Tat protein produced by the pNL1.4.7 cDNA expression plasmid, while the lower band corresponds to the truncated one-exon Tat protein produced by the HLtat cells. pNL1.4A.7 produced both Rev and *nef*, while pNL1.5.7 could produce only Nef. (B) The three *nef* cDNA expression plasmids pNL1.5.7, pNL1.2.5.7, and pNL1.3.5.7 (structures shown at the top) were transfected into HLtat cells, and their protein products were analyzed by immunoprecipitation with preimmune serum (P) and anti-Nef (N) antiserum. Similar amounts of Nef protein were produced from the three cDNAs.
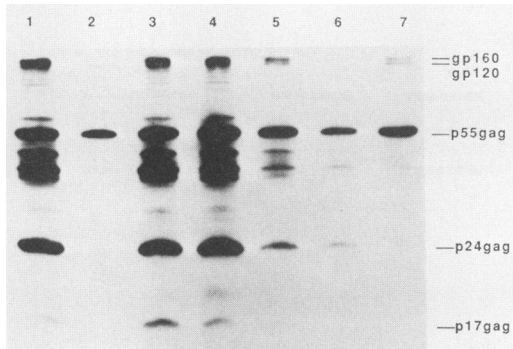
FIG. 6. Functional assay for the expression of Rev protein by the different cDNA clones. The HLfB cell line containing the *rev* mutant fB was transfected with the various cDNA clones, and cell lysates were analyzed for p24^{gag} production by Western immunoblotting as described in Materials and Methods. Lanes: 1, transfection of HLfB cells with the Rev-expressing plasmid pL3crev as a positive control; 2, HLfB cells transfected with 5 μg of pNL1.5.7; 3 and 4, HLfB cells transfected with 5 μg of pNL1.4B.7 or pNL1.4A.7, respectively (*trans*-complementation by Rev produced by pNL1.4B.7 and pNL1.4A.7 completely restored the wild-type phenotype of the mutant virus): 5 and 6, transfection with 15 and 5 μg of pNL1.4.7, respectively (lower levels of p24^{gag} were detected, indicating that pNL1.4.7 produced only very low levels of Rev protein); 7, transfection with salmon sperm DNA. HLfB cells do not produce p24^{gag} or p17^{gag} proteins.

expression to a similar extent in these cells, suggesting that high levels of functional Tat protein were produced from all cDNAs. As expected, vectors expressing *rev* or *nef* cDNAs were unable to activate *cat* expression, since they all lack an intact *tat* gene (data not shown).

To assay for Rev function, we transfected the cDNA expression plasmids into HLfB cells (Mermer et al., in press), which contain stably integrated copies of a *rev* mutant molecular clone of HIV-1. These cells produced low levels of p55^{gag} and Env proteins (Fig. 6, lane 7), but did not process p55^{gag} to p24^{gag} and p17^{gag} and did not produce virus particles. As shown in lane 1 of Fig. 6, complementation by Rev protein after transfection of the Rev-producing plasmid pL3crev resulted in an increased production of p55^{gag} and Env proteins, as well as the production of p24^{gag} and p17^{gag}. We used this complementation assay to study the expression of Rev protein by the different cDNAs. Both pNL1.4B.7 and pNL1.4A.7 were able to complement the *rev* defect in HLfB cells (Fig. 6, lanes 3 and 4, respectively), indicating production of high levels of functional Rev protein. All six different 4A- or 4B-containing cDNAs complemented equally well (data not shown). Transfection of pNL1.4.7, which contains both *tat* and *rev* overlapping reading frames, resulted in very low levels of complementation (lanes 5 and 6). When 15 μg of plasmid pNL1.4.7 was used in the transfection (lane 5) instead of 5 μg (lane 6), higher levels of p24^{gag} and Env proteins were detected, indicating that low levels of Rev protein are produced from the *tat* mRNAs. However, these levels were too low to fully restore the wild-type phenotype of the *rev* mutant provirus in HLfB cells. Therefore, efficient production of Rev requires the presence of the *rev*-specific mRNAs. As expected, Nef-producing pNL1.5.7 did not complement the defect in HLfB cells, since it lacks the *rev* AUG (lane 2).

DISCUSSION

A surprisingly large number of mRNAs are produced by HIV-1. The three regulatory proteins Tat, Rev, and Nef are
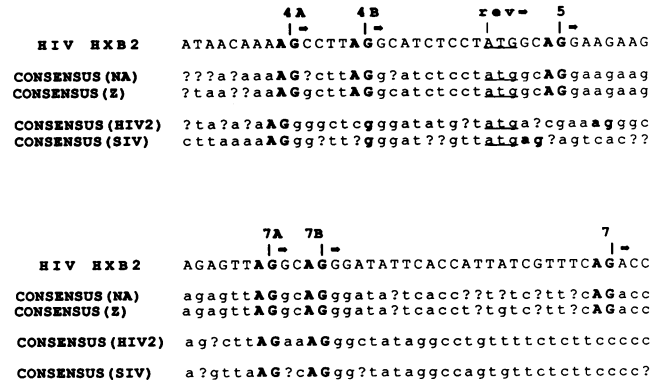

FIG. 7. Sequence comparisons of the regions of splice acceptors 4A, 4B, 5, 7A, 7B, and 7. The consensus sequences in the regions of splice acceptors 4A, 4B, 5, 7A, 7B, and 7 are shown for North American (NA) and Zairean (Z) HIV-1 isolates, as well as for HIV-2 and SIV as compiled by Myers (44). The nonconserved positions are indicated with ?. Splice acceptors 4A and 4B are conserved among the HIV-1 isolates, while only 4A is conserved in HIV-2 and SIV. Both splice acceptors 7A and 7B are conserved among HIV-1 isolates as well as in HIV-2 and SIV. The invariable dinucleotides of the acceptor sites (i.e., AG) are shown in boldface type. The *rev* initiator ATG is underlined.

produced from 12 mRNAs. Each protein is produced from a group of mRNAs consisting of at least three mRNAs differing in the leader sequence preceding the coding exons. This is a result of splicing to the small noncoding exons 2 and 3, located in the 5' part of the virus (Fig. 1). Structural and functional assays did not reveal any significant difference between the Tat-expressing cDNAs or between the Rev- or the Nef-expressing cDNAs. These results differ from those of Muesing et al. (43), who reported that 1.3.4.7 *tat* cDNA transactivated the HIV-1 LTR five times better than 1.4.7 cDNA.

The splice acceptors of exons 2 and 3, at positions 4459 and 4936, respectively, are apparently utilized to produce mRNAs encoding Vif and Vpr proteins. The predicted structures of the *vif* and *vpr* mRNAs are shown in Fig. 3B. The production of these mRNAs could potentially decrease the levels of expression of the proteins encoded downstream of *vif* and *vpr*, e.g., *tat*, *rev*, *vpu*, *env*, and *nef*. A possible role for the splicing with the splice donors defining exons 2 and 3 might be the production of high levels of mRNAs expressing these downstream proteins. In this regard, it is noteworthy that mRNAs containing exons similar to 2 and 3 in HIV-1 are produced by other lentiviruses such as SIV$_{MAC}$ (9) and visna virus (11).

Four new splice sites were identified by cloning and sequencing, and the corresponding exons were named 4A, 4B, 7A, and 7B. The 5' splice sites generating exons 4A and 4B are positioned only six nucleotides apart, after the *tat* AUG and before the *rev* AUG. Thus, utilization of either splice acceptor 4A or 4B allows expression of *rev* but not *tat*. A similar mechanism to generate *rev* mRNA(s) has been proposed for the SIV$_{MAC}$ virus isolate by Colombini et al. (9). These investigators identified a cDNA clone that uses a splice acceptor between the *tat* and *rev* AUGs and is believed to represent the Rev-producing mRNA. The observation that cDNAs containing exon 4A or 4B were cloned with the same frequency after PCR amplification indicates similar use of both splice acceptors. In addition, direct detection of exons 4A and 4B in infected or transfected cells by S1 nuclease analysis revealed similar levels of mRNAs

TABLE 1. Protein production of four different HIV-1 mRNAs

mRNA	Coding capacity	Protein production		
		Tat	Rev	Nef
1.4.7	tat, rev, nef	+	+/−	+/−
1.4A.7	rev, nef	−	+	+
1.4B.7	rev, nef	−	+	+
1.5.7	nef	−	−	+

containing these exons (Felber et al., submitted). Although the purpose of using two splice acceptors in this region is unclear, it is noteworthy that both splice acceptors are conserved among different HIV-1 isolates (Fig. 7). In addition, the splice acceptor 4A is also well conserved in the distantly related lentiviruses HIV-2 and SIV. The splice acceptor site for exon 4B is conserved only among the different HIV-1 isolates. The newly identified splice acceptors were also compared with the consensus splice acceptor sequence (59). The splice acceptor site 4B is in good agreement with the consensus splice acceptor sequence, while 4A is not.

The splice acceptors of exons 7A and 7B are located before exon 7, the last exon of HIV-1 (Fig. 2). These acceptors should be able to generate mRNAs producing isoforms of Tat and Rev or hybrid proteins between the first exon of Tat and Env or the first exon of Rev and the second exon of Tat. Both splice acceptors are conserved in HIV-1 isolates, as well as in HIV-2 and SIV (Fig. 7). Although the biological significance of these mRNAs remains to be investigated, it is noteworthy that an alternative splice acceptor has also been found in the last exon of the SIV$_{MAC}$ genome (64). This further underlines the similar organization of the lentiviruses.

Although the splice sites producing exon 6D are not conserved in all HIV strains, it is interesting that this exon was present in many small multiply spliced mRNAs. This observation indicates that the generation of one splice site might result in many new alternatively spliced mRNAs. This might be an additional mechanism by which lentiviruses diverge at a high rate. Exons 6D, 7A, and 7B could generate several different mRNAs by alternative splicing. Assuming that exon 6D could be spliced to all 12 mRNA species reported here, a total number of 24 differentially spliced small mRNAs would be generated. Furthermore, if the splice acceptors for exons 7A and 7B are utilized in addition to exon 7, a total of 72 differentially spliced small HIV-1 mRNAs would be generated. We have previously shown that one of the 6D-containing mRNAs produces a hybrid protein, Tev (7). The protein products of other exon 6D-containing mRNAs have also been studied (7). Although the significance of these mRNA species for the virus remains to be determined, identification of the alternative splice sites further underlines the complexity of HIV-1 splicing. The acquisition of regulatory factors such as Rev, which allows the production of appropriate quantities of structural mRNAs, may have allowed the incorporation of additional splice signals and the generation of many alternatively spliced mRNAs. Alternative splicing may offer an evolutionary advantage to the virus, since proteins are produced from more than one mRNA. Also, the plasticity of the genome may allow new combinations to arise and enable viral adaptation to a new environment.

Results of the functional studies of the HIV-1 cDNA clones shown in Fig. 5 and 6 and summarized in Table 1

revealed that tat mRNAs produced very low levels of Rev and Nef, while rev mRNAs produced high levels of both Rev and Nef proteins. These data are in agreement with the scanning model for initiation of translation (34, 35), according to which the ribosome binds at the 5' end of the mRNA and scans the RNA for a suitable initiation site. Initiation at the tat AUG appears to be very efficient, allowing the ribosome to read the downstream AUGs for rev and nef only in rare instances. This results in high production of Tat but only low production of Rev and Nef (Fig. 5A). In contrast, the rev AUG seems to allow readthrough of the ribosome to a much higher degree than the tat AUG, resulting in high levels of both Nef and Rev proteins expressed from the rev mRNAs (Fig. 5A).

Since tat mRNAs are very poor producers of Rev protein, specific rev mRNAs containing exon 4A or 4B are essential for production of sufficient amounts of Rev. Rev produced from mRNAs containing either exon 4A or 4B was sufficient to restore virus expression from the rev mutant provirus integrated in HLfB cells (Fig. 6). These data are in disagreement with the results presented by Sadaie et al. (54), who reported that a derivative of HXB2 containing a point mutation at the exon 4B splice site displayed a rev mutant phenotype after transfection into COS cells (mutant M43 [54]). Our data show that HXB2 can generate another equally potent rev mRNA by using the adjacent splice acceptor, 4A. Therefore, mutant M43 should also produce Rev. Accordingly, we were able to show that M43 produced functional levels of Rev protein by using the complementation assay described above (data not shown). We also were able to immunoprecipitate Rev from M43-transfected HeLa cells. We concluded that M43 is a rev-deficient but not rev-minus mutant.

The multiply spliced mRNAs lack the RRE element and are present in high levels in the absence of Rev (17, 28). The cloning and characterization of the majority, if not all, of the multiply spliced HIV-1 mRNAs allowed the generation of specific probes for the study of the expression of the individual species by S1 nuclease analysis (Felber et al., submitted). This is necessary in order to quantitate the different mRNAs present in infected cells, since PCR amplification results in a nonlinear amplification of target cDNAs. Concerning the reliability of the PCR technique, repetitive cloning and sequencing of the same cDNAs from different cDNA libraries created by PCR did not reveal any sequence aberrations. We therefore believe that PCR, as applied here, is a reliable technique for the qualitative analysis of cDNAs.

We have shown that HIV-1 produces one group of mRNAs for each regulatory protein Tat, Rev, and Nef, presumably allowing high levels of expression of each of these proteins by the virus. It is interesting that the Nef protein is produced in high amounts from its own group of three monocistronic mRNAs as well as from the six bicistronic rev mRNAs. Although HIV-1 produces several bicistronic mRNAs, it does not depend on the expression of more than one protein from each of the mRNAs producing Tat, Rev, and Nef. This is unlike the organization of the human T-cell leukemia viruses (45), which are proposed to rely on a single doubly spliced mRNA for the production of both Tax and Rex regulatory proteins.

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