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Structure and Expression of *tat*-, *rev*-, and *nef*-Specific Transcripts of Human Immunodeficiency Virus Type 1 in Infected Lymphocytes and Macrophages

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Primary RNA transcripts from the human immunodeficiency virus type 1 (HIV-1) are processed into mature mRNA by a complex series of splicing events. Viral structural proteins and reverse transcriptase are translated from unspliced or singly spliced transcripts. Proteins which control virus replication, including *tat*, *rev*, and *nef*, are translated from transcripts which are the product of multiple splicing. We have analyzed the composition and relative abundance of the latter transcripts in long-term infected cell lines and in acutely infected peripheral blood cells by amplification with the polymerase chain reaction (PCR) followed by Southern blot, molecular cloning, and DNA sequence analyses. In H9 cells chronically infected with the HIV-1 strain HTLV-III$_B$, the predominant of the three kinds of transcripts is those coding for *nef*. Transcripts with coding potential for *rev* constituted an intermediate fraction of those analyzed, while those for *tat* accounted for only a small minority. A similar pattern was observed with Southern blots of PCR-amplified transcripts from peripheral blood lymphocytes acutely infected with HTLV-III$_B$. The same general pattern was also observed with PCR-amplified transcripts from peripheral blood monocyte-macrophages infected with an HIV-1 strain (BA-L) able to grow to high titers in macrophages. In these cells, however, the apparent major form of *nef* transcript contained only the first and third exons of the multiply spliced transcripts and appeared to be generated by either a single or a triple splicing mechanism. As with lymphocytes, *tat*-specific mRNAs were by far the least abundant. It thus appears that different cell types infected with different strains of HIV-1 maintain a similar balance of expression in which transcripts for *nef* vastly predominate over those for *tat* and that those for *rev* are intermediate in abundance.

The primary full-length RNA transcript of the human immunodeficiency virus type 1 (HIV-1), the etiologic agent of the acquired immune deficiency syndrome, is processed to mature mRNA species by a complex series of splicing events (2, 16). As with other retroviruses, full-length RNA is translated into the *gag* core structural proteins and also, by a translational frameshift, the *pol* products (12), while the *env* mRNA is generated by a single splicing event. There are also a number of smaller, multiply spliced transcripts present in HIV-1-infected cells which code for some of the other genes of HIV-1, including the regulatory genes *tat*, *rev*, and *nef* (1, 2, 16). These transcripts consist of three or more exons, and their expression and that of the *gag-pol* and *env* mRNAs are differentially regulated. This regulation is mediated by the *rev* gene, which appears to bind to target sequences within the *env* gene and facilitate transport of mRNA from the nucleus before multiple splicing occurs (5, 7, 10, 11, 14, 15). In the absence of *rev*, most viral RNA undergoes multiple splicing before exiting the nucleus, and little synthesis of viral structural proteins is observed (6). *tat* acts, possibly at several different levels, to increase the overall level of viral RNA (4, 6, 22), while *nef* has been reported to have a negative effect on the rate of viral replication (13, 17).

The balanced expression of these multiply spliced transcripts is thus critical in regulating virus replication and expression. Because of similarities in size, however, the analysis of their relative expression levels by Northern (RNA) blots is not feasible. We therefore undertook to use the polymerase chain reaction (PCR) to amplify reverse transcripts of selected regions of these mRNAs. The amplified fragments were then analyzed by Southern blotting, molecular cloning, and DNA sequence analyses in order to characterize their composition and relative abundance.

MATERIALS AND METHODS

Cells and viruses. The H9 cell line infected by the HTLV-III$_B$ strain of HIV-1 (18) was used as a source of RNA from chronically infected cells. Virus from this cell line was used to infect fresh phytohemagglutinin-stimulated peripheral blood lymphocytes as described previously (18). Infected cells were harvested 48 h after infection. The BA-L strain of HIV-1, which has a high replicative ability in macrophages (8), was used to infect fresh peripheral blood monocyte-macrophages as described elsewhere (8). Infected monocyte-macrophages were harvested after 12 to 14 days in culture. Control cells included uninfected H9 cells, uninfected monocyte-macrophages after 12 days of culture, and uninfected peripheral blood lymphocytes 48 h after activation with phytohemagglutinin (5 μg/ml).

Nucleic acids. RNA was purified from 10^7 to 10^8 cells by digestion with proteinase K (100 μg/ml, 42°C, 90 min) in the presence of 1% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.4)–5 mM EDTA. The RNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) (pH 7.4) and then precipitated overnight at −20°C by the addition

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of sodium acetate to 0.45 M, followed by the addition of 2.5 volumes of ethyl alcohol (EtOH). RNA from monocyte-macrophages was prepared by the guanidine isothiocyanate method (3). The amount and quality of RNA were estimated by measuring the A_{260} and A_{280} and by analyzing a sample on a formaldehyde-agarose gel and comparing the relative intensities of the bands of 18S and 28S rRNAs visualized by staining with ethidium bromide.

For PCR analyses, the RNA was first copied with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 100-μl reaction mixtures containing the following: 10 mM Tris hydrochloride (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM each dATP, dCTP, dGTP, and TTP; 1 μg of antisense primer; 1 μg of RNA; and approximately 15 U of reverse transcriptase. After 1 h at 37°C, 1 μg of sense primer and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) were added and the mixture was heated to 94°C for 4 min to inactivate the reverse transcriptase. The PCR was carried out by using a DNA Thermal Cycler (Perkin-Elmer Cetus) under conditions specified by the manufacturer. Repetitive cycles consisted of 1 min 45 s of denaturation at 94°C, 1 min 45 s of annealing at 43°C, and 1 min 30 s of extension at 72°C. After 30 cycles, a final 4 min of extension at 72°C was carried out.

Samples of the PCR reaction mix were analyzed by electrophoresis on 3% gels containing 3 parts NuSieve GTG and 1 part SeaKem agarose (FMC BioProducts, Rockland, Maine) followed by capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) to a NyTran membrane (Schleicher & Schuell, Inc., Keene, N.H.). DNA was immobilized by cross-linking to the membrane with UV light in a Stratalinker (Stratagene, La Jolla, Calif.) and hybridized with the indicated oligonucleotide probes. Prehybridization was with 6× SSC–10× Denhardt solution–20 μg of tRNA per ml–50 μg of denatured salmon sperm DNA per ml for 4 h at 37°C. Hybridization was carried out for 1 h at room temperature in 6× SSC containing 1% sodium dodecyl sulfate and 1×10^6 to 2×10^6 cpm of oligonucleotide probe labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) to a specific activity of $\sim 10^9$ cpm/μg. The hybridized blots were washed several times at temperatures ranging from 37 to 55°C, depending on the probe, in 6× SSC–0.1% sodium dodecyl sulfate and developed by autoradiography at −70°C with X-Omat film (Eastman Kodak Co., Rochester, N.Y.) in a cassette, using a Quanta III intensifying screen.

For cloning experiments, amplified fragments were separated from unincorporated primers and primer dimers by electrophoresis in 1.5% agarose gels. The fragments were recovered by electrophoresis onto a DEAE membrane (NA-45; Schleicher & Schuell), elution with 1 M NaCl–1 mM EDTA–10 mM Tris hydrochloride (pH 8), and precipitation with 2 volumes of EtOH. After the precipitate was washed with 70% EtOH, the amplified fragments were digested with the indicated restriction endonucleases under conditions specified by the manufacturer (Boehringer-Mannheim Biochemicals, Bethesda Research Laboratories, or New England BioLabs, Inc., Beverly, Mass.), extracted once with phenol-chloroform and once with chloroform alone, and then reprecipitated with salt-EtOH. After a washing with 70% EtOH, a sample containing one-third of the purified amplified fragments (~5 to 20 ng) was ligated with T4 DNA ligase (Bethesda Research Laboratories) for 4 to 16 h at 12°C in a buffer supplied by the manufacturer into an M13 vector (Phagescript SK; Stratagene) (50 ng) cut with the appropriate restriction endonucleases. A small portion (1 to 2%) was transfected into competent host cells (DH5α F′; Bethesda Research Laboratories) and plated overnight. The resultant clones were analyzed by hybridization with the oligomer probes as described below and by DNA sequence analyses using the dideoxy termination method (21).

RESULTS

PCR amplification of *tat*, *rev*, and *nef* mRNAs. The strategy for analyzing the relative levels of expression of multiply spliced HIV-1 mRNAs was to first synthesize cDNA from RNA from infected cells, using the antisense primer shown in Fig. 1, and then to amplify the cDNA by the PCR technique, using the original primer and the sense primer shown in Fig. 1. The PCR extension time was sufficiently short (1 min 30 s) to preclude significant amplification of the approximately 8-kilobase segment present between these two primers on viral DNA. The mRNAs for *tat*, *rev*, and *nef* would be expected to contain less than 1 kilobase between the primer binding sites. The amplified mRNAs were then analyzed by gel electrophoresis and Southern blotting, using specific oligonucleotide probes. Probes specific for the *nef* plus *rev* plus *tat* middle exon (Fig. 1, D3), *rev* plus *tat* (D2), and *tat* alone (D1) were designed on the basis of previous studies of HIV-1 mRNA structure (2, 16, 20). The probes were hybridized in the order D1-D2-D3 to detect, respectively, *tat*, *rev*, and *nef*. Probes were also used for the two small upstream exons reported by Muesing et al. (16) (Fig. 1, exons B and C) and the 3′-terminal exon (E).

Southern blots of amplified cDNA from H9 cells chronically infected with the HTLV-III$_B$ strain of HIV-1 were hybridized with the different probes. The most prominent band detected with the *tat*-specific probe was approximately 580 base pairs (bp). This band sometimes appeared as a closely spaced doublet. The main *rev*-specific band was at 400 bp and was only slightly resolved from the main *nef*-specific band at 380 bp. Other, faint bands of larger sizes hybridizing with these probes also hybridized with probes for the small upstream exons B and C. A smaller band, which was sometimes evident by ethidium bromide staining but which failed to hybridize with any of the previous probes, was labeled with a probe for exon E. By the pattern obtained by staining with ethidium bromide, the *nef*-specific band appeared by far the most abundant, and the *tat*-specific band was by far the least abundant. This was further demonstrated by hybridization with the exon E probe common to all the amplified bands (Fig. 2, lane a). Amplified RNA from acutely infected peripheral blood T lymphocytes gave a pattern substantially similar to that from the infected H9 cells (not shown).

The pattern of expression of the small multiply spliced mRNAs in acutely infected macrophages was also examined. The BA-L strain of HIV-1, which is highly replicative in macrophages, was used to infect fresh peripheral blood monocyte-macrophages. RNA was purified 12 to 14 days after infection, and PCR-amplified cDNAs were prepared and analyzed by Southern blotting and molecular cloning in M13. The Southern blots differed from those of the H9 and lymphocyte cDNA primarily by the presence after ethidium bromide staining of a predominant band of a relatively small size (~320 bp) which hybridized only with the probe for exon E (Fig. 2, lane c) and a concomitant decrease in the relative abundance of the 380-bp *nef*-specific band.

DNA sequence analyses of amplified cDNAs. In order to confirm the inferred structures of the amplified cDNAs, the

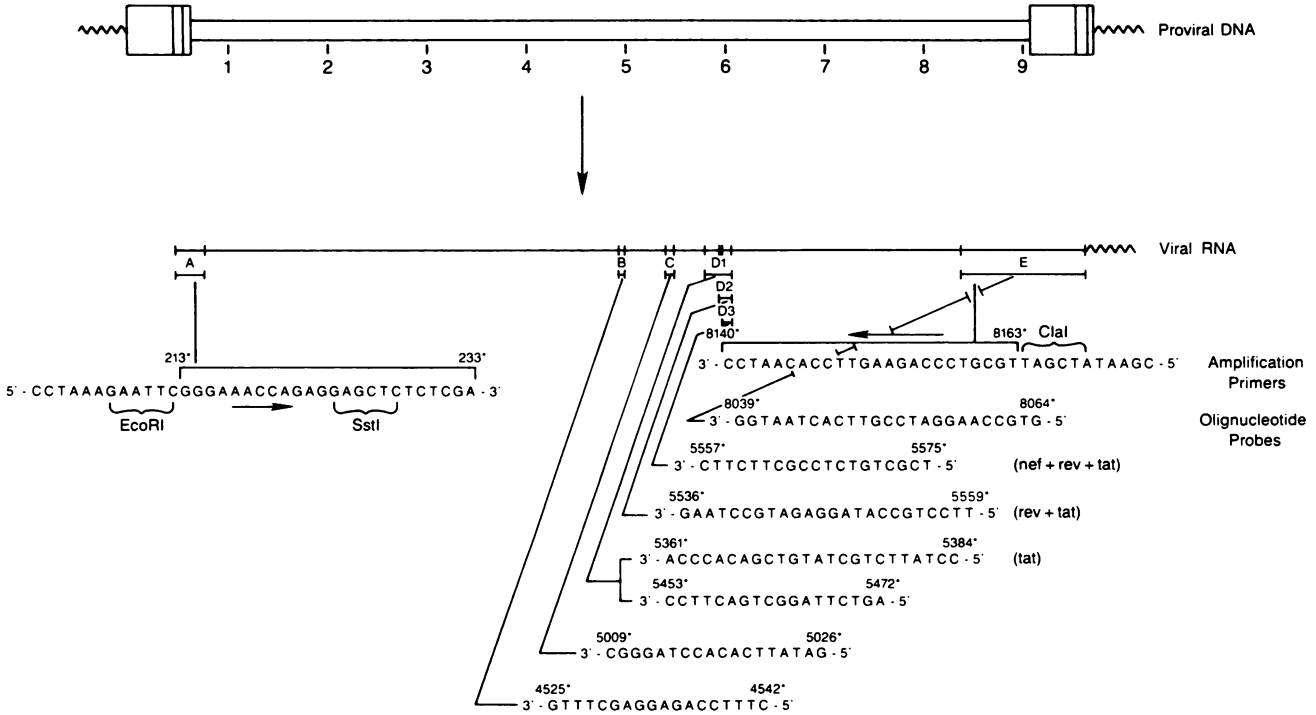


FIG. 1. Oligonucleotide primers and probes. The oligonucleotides whose sequences are indicated were synthesized on a model 381A DNA synthesizer (Applied Biosystems). All PCR amplifications were performed by using the base 8039 to 8164 oligomer as the primer for the reverse transcription step. The base 213 to 233 primer was added with the *Taq* polymerase for the amplification cycles. Exons D1, D2, and D3 represent, respectively, the first coding exons for *tat* and *rev* and the middle exon of the doubly spliced *nef* mRNA. The probes for bases 5361 to 5384 or 5453 to 5472, 5536 to 5539, and 5557 to 5575 were used in sequential hybridizations to amplified DNA to detect *tat*, *rev* plus *tat*, and *nef* plus *rev* plus *tat*, respectively. The small upstream exons (B and C) were detected with the probes for bases 4525 to 4542 and 5009 to 5026. The second coding exon for *rev* and *tat* and the first coding exon for *nef* (exon E) were detected with the probe for bases 8039 to 8064. The boxes on the proviral DNA represent the long terminal repeat regions. The numbering system is that of Ratner et al. (19).

unincorporated primers were removed by gel electrophoresis as described in Materials and Methods and the PCR product was digested with *Cla*I and *Eco*RI and cloned into the same sites in the polylinker of M13 Phagescript (Stratagene). Plaques hybridizing to combinations of probes corresponding to patterns seen with different bands on the Southern blots were grown and analyzed by DNA sequencing, using T tracking. Samples yielding identical T tracks were grouped, and individual members of each group were analyzed by standard DNA sequencing.

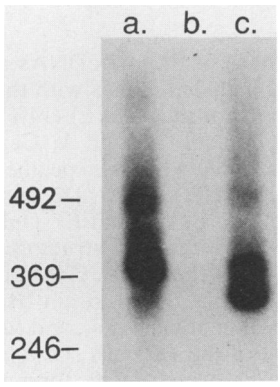
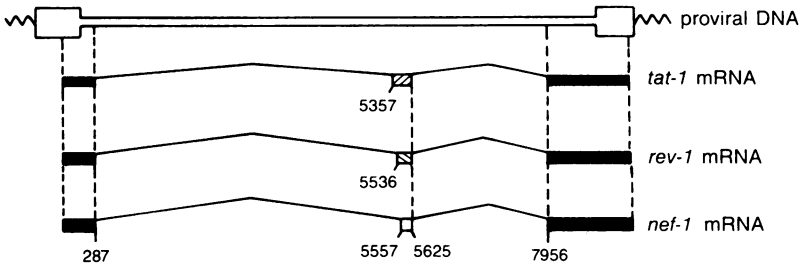


FIG. 2. Southern blot analysis of cDNAs of multiply spliced HIV-1 mRNAs. RNA was purified from H9 cells infected with HTLV-III$_B$ (lane a), uninfected H9 cells (lane b), or fresh peripheral blood monocyte-macrophages infected with HIV-1 (BA-L) (lane c). Fifteen microliters from standard PCR reactions was analyzed by Southern blotting as described in Materials and Methods and hybridized to the probe for exon E (Fig. 1). The positions of DNA markers in a 123-bp ladder (Bethesda Research Laboratories) are indicated (in base pairs) at the left.

DNA sequence data confirmed the hypothesis that the 580-, 400-, and 380-bp bands represented mRNAs containing three exons and able to code for *tat*, *rev*, and *nef*, respectively (represented at the top of Fig. 3). The data, consistent with an earlier report (20), revealed a mRNA distinct from that of *tat* and able to code for *rev*. In addition, they allowed the precise assignment of its splice junction. Base 287 (using the numbering system of Ratner et al. [19]) was joined to base 5536, 15 bases upstream from the *rev* initiation codon (Fig. 3). Four of four cDNAs sequenced had the same splice junction. Two *tat* cDNAs sequenced were identical to the *tat* cDNA previously reported (2, 16).

The DNA sequence data also indicated a great deal of diversity in transcripts able to code for *nef*. Two of these structures (Fig. 4) have been previously reported (2, 16). One consisted of three exons (A, D3, and E). The first exon was joined at base 287 to the middle exon at base 5557, and the middle exon was joined at base 5635 to the third exon, which contains the *nef*-coding region, at base 7956. A second structure was identical except for the presence of a small exon (C), derived from bases 4969 to 5043 immediately upstream from the *vpr* reading frame, between the A and D3 exons. The other three *nef*-specific cDNAs analyzed, also



Coding exon 1 of *rev-1* and *tat-1*

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5342                    tat SA
                          V
GTTTATCCATTTTCAGAATTGGGTGTCGACATAGCAGAATAGGCGTTACTCGACAGAGGA

GAGCAAGAAATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAGGAAGTCAG
       tat   M   E   P   V   D   P   R   L   E   P   W   K   H   P   G   S   Q

CCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGT
 P   K   T   A   C   T   N   C   Y   C   K   K   C   C   F   H   C   Q   V   C

                                                nef SA
                                                  V
TTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGA
 F   I   T   K   A   L   G   I   S   Y   G   R   K   K   R   R   Q   R   R   R
                             rev   M   A   G   R   S   G   D   S   D   E   D
                                                        SD      5632
                                                         V      |
CCTCCTCAAGGCAGTCAGACTCATCAAGTTTCTCTATCAAAGCAGTAAGTA
 P   P   Q   G   S   Q   T   H   Q   V   S   L   S   K   Q
   L   L   K   A   V   R   L   I   K   F   L   Y   Q   S
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***rev-1* mRNA**

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                                                        287 SJ 5536
                                                           V
TCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGCTTAGGCATCTCCT

ATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTCATCAAG
 M   A   G   R   S   G   D   S   D   E   D   L   L   K   A   V   R   L   I   K
                5625 SJ 7956
                   V
TTTCTCTATCAAAGCAACCCACCTCCCAACCCCGGGGGGACCCGACAGGCCCGAAGGAAT
 F   L   Y   Q   S   N   P   P   P   N   P   G   G   T   R   Q   A   R   R   N

AGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCC
 R   R   R   R   W   R   E   R   Q   R   Q   I   H   S   I   S   E   R   I
```

FIG. 3. Nucleotide sequence and splice junctions of *rev* cDNA clones from HTLV-III$_B$. The structures of doubly spliced mRNAs for *tat*, *rev*, and *nef* are shown at the top. The middle exons of previously characterized doubly spliced mRNAs for *tat* and *nef* are shown, with the splice junctions indicated by base number (19). The nucleotide sequences of four different clones of *rev*-specific cDNA are shown at the bottom.

shown in Fig. 4, lacked the middle (D3) exon and differed slightly in their splice junctions. One cDNA consisted of exon A joined at base 287 to base 7956 of exon E, while another was spliced from base 269 to base 7956. The third cDNA was spliced from approximately base 325 to base 8013; the precise junction is impossible to assign because of a repeat sequence (GGAGAGAGA) present at both bases 325 and 8013. Only one example of this cDNA was found, and it may therefore represent a cloning or PCR artifact.

Relative levels of *tat*-, *rev*-, and *nef*-specific transcripts. In order to better quantitate the relative levels of expression of each of these mRNAs, we analyzed a large number of M13 clones of amplified RNA from the infected H9 cells. The clones were plated out and hybridized with the same probes used for the Southern blots. This procedure, together with the DNA sequence data, allowed the analysis and enumeration of individual amplified mRNAs. The results (Fig. 5) agreed with the Southern blot results, suggesting that *nef*-specific transcripts are the most abundant of these small viral mRNAs.

Of a total of 169 clones examined, 134 (79%) had structures similar to those of the *nef* cDNAs analyzed by DNA sequencing. They included clones with three exons (A, D3, and E) (60%), two exons (A and E) (14%), and four exons, either A, B, D3, and E (2%) or A, C, D3, and E (4%). Thirty-two clones (19%) were *rev* specific and included two clones with four exons (A, B or C, D2, and E) and one clone with five exons (A, B, C, D2, and E). Only three *tat* clones (2%) were observed; all had the structure A-D1-E.

A similar analysis of the cDNA clones from macrophages infected with a different HIV-1 strain (BA-L) showed that the relative expression of *nef*-, *rev*-, and *tat*-specific mRNAs approximated that of the H9 cells. Of a total of 355 cDNA clones examined, 77% were specific for *nef*, 20% for *rev*, and 3% for *tat* (Fig. 5). In contrast to the H9 clones, however, the majority of the *nef*-specific clones (204 of 274) contained only two exons (A and E). From these data, it is apparent that the two exon *nef* cDNAs are represented by the intense 320-bp band observed in Southern blots of cDNA from infected macrophages. None of the *nef*-, *rev*-, or *tat*-specific clones in infected macrophages were found to contain either of the upstream exons B or C.

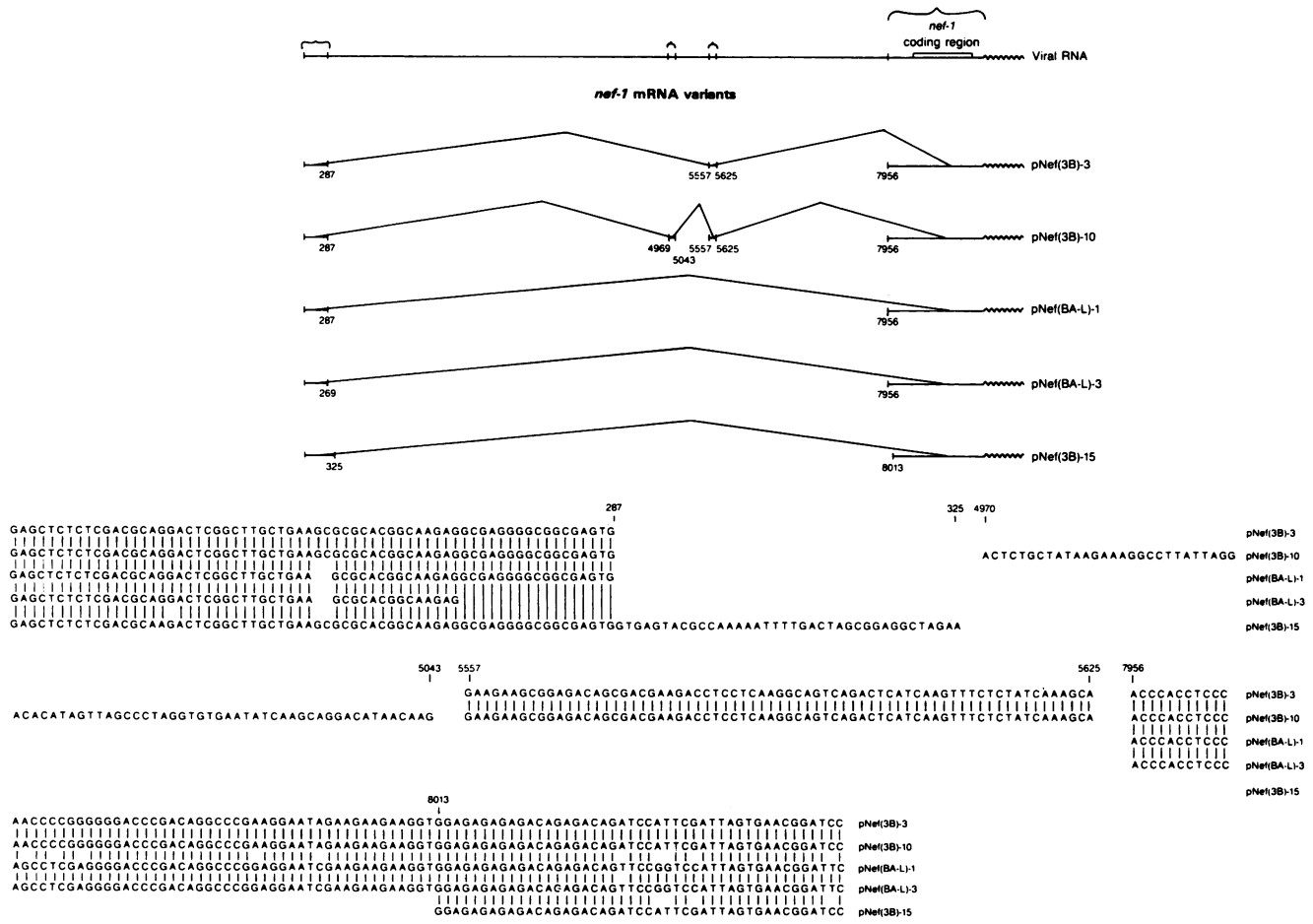

FIG. 4. Structures of mRNAs potentially encoding *nef*. M13 clones of *nef*-specific cDNA from either HTLV-III$_B$ or HIV-1 (BA-L) were selected from PCR-generated libraries on the basis of a lack of hybridization to the *tat*- and *rev*-specific oligomers and the ability to hybridize to the probe for exon E (Fig. 1). The DNA sequences of selected cDNA clones, determined as described in Materials and Methods, are shown above. Clones pNef (3B)-3, -10, and -15 were from HTLV-III$_B$ and pNef(BA-L)-1 and -3 were from HIV-1 (BA-L). The exon structures of the corresponding mRNAs inferred from the cDNA nucleotide sequences are shown above. The wavy line represent the poly(A) tracts of the viral RNAs.

DISCUSSION

We have used the PCR technique to selectively amplify the small, multiply spliced mRNA species from HIV-1-infected lymphocytes and monocyte-macrophages and analyze the relative levels of *tat*-, *rev*-, and *nef*-specific transcripts. We base our conclusions on the assumption that all transcripts are amplified with equal efficiency. This assumption does not seem unreasonable in view of the fact that all the transcripts described here are amplified with the same primer pairs. The amplified RNA was analyzed by Southern blotting and sequential hybridization, using oligonucleotide probes specific for *tat*, *tat* plus *rev*, and *tat* plus *rev* plus *nef*.

Southern blots of amplified RNA from H9 cells chronically infected with the HTLV-III$_B$ strain of HIV-1 showed that the sizes of the predominant amplified units for *tat*, *rev*, and *nef* were 580, 400, and 380 bp, respectively. This would correspond to original transcript sizes of approximately 1,820, 1,640, and 1,620 bp, respectively, not including the poly(A) tract. Ethidium bromide staining of gels of the amplified DNA suggested that *nef* was by far the most abundant transcript and that the *tat* transcript was present at relatively low levels. A similar pattern was observed when mRNAs from fresh peripheral blood lymphocytes infected with HTLV-III$_B$ and harvested 48 h after infection were similarly analyzed.

The identities of the putative *tat*, *rev*, and *nef* transcripts were verified by cloning the amplified mRNAs into M13, selecting individual clones on the basis of their patterns of hybridization to the different probes, and determining their DNA sequences. The nucleotide sequences of the 580- and 380-bp fragments corresponded to those of previously described cDNAs synthesizing *tat* and *nef*, respectively (1). Each fragment is derived from a doubly spliced mRNA containing three exons. The 400-bp fragment represents a doubly spliced mRNA whose upstream splice junction is just upstream of the initiation codon for *rev*. This finding confirms an earlier report (20) of a unique mRNA for *rev* which is distinct from that for *tat* and maps the splice junction as being from base 287 to base 5536, following the numbering system of Ratner et al. (19).

In addition, several other apparent forms of *rev* and *nef* mRNAs were found. They contained four or five exons and included one or both of two small upstream exons described by Muesing et al. (16). The more upstream of these exons is just 5′ to the initiation site for *vif*. The more downstream of the two lies just 5′ of the initiation site for *vpr*. It is possible

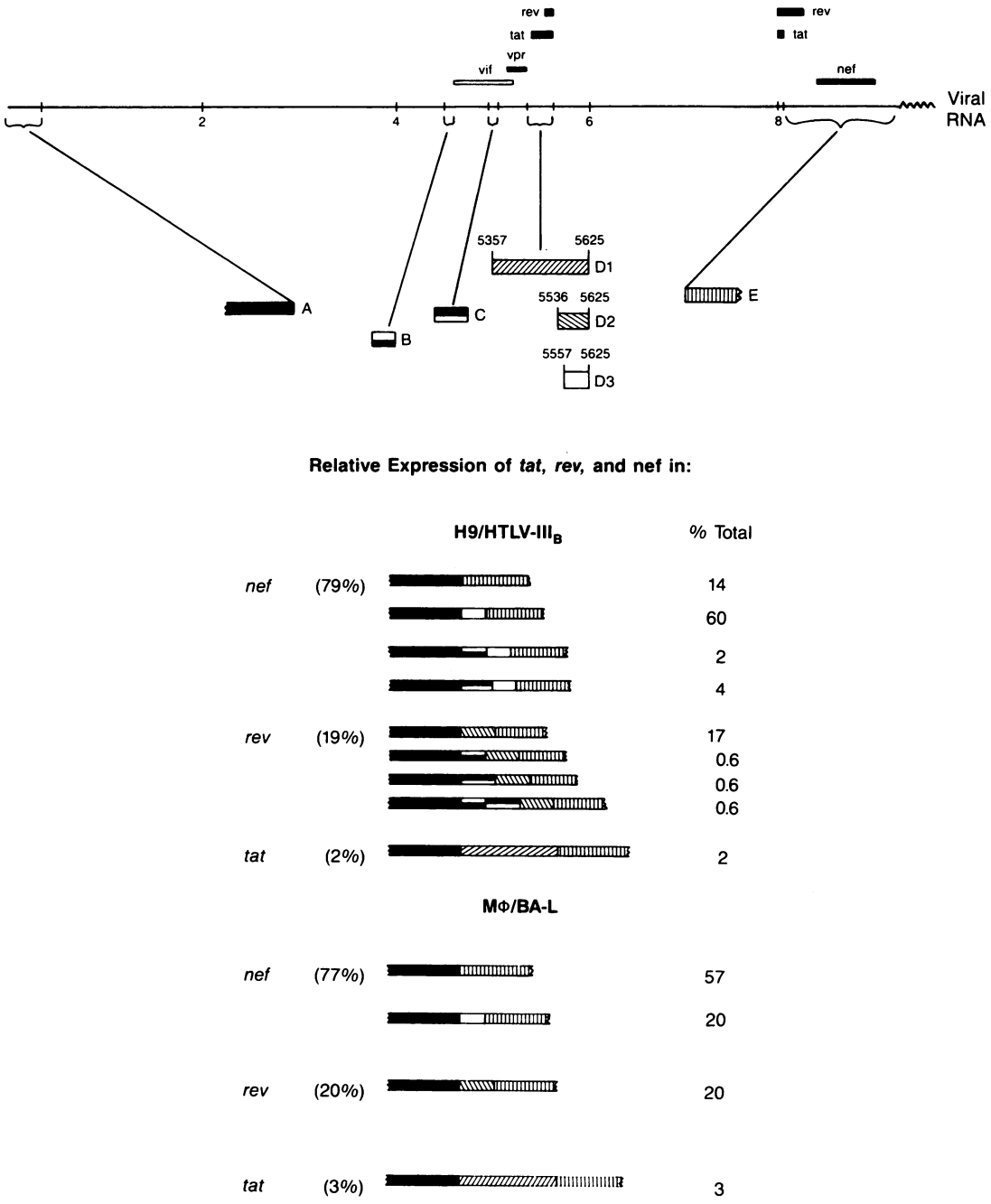

FIG. 5. Relative abundance of *tat*-, *rev*-, and *nef*-specific transcripts in HIV-1-infected T cells and macrophages. M13 clones were plated and characterized by hybridization as described in Materials and Methods to a series of oligonucleotide probes specific for different exons, represented by open, closed, partially closed, or hatched bars, as indicated at the top. The percentage of the total clones analyzed which are specific for *nef*, *rev*, and *tat* is given on the left; the percentage of the total transcripts with each of the indicated structures is given on the right. The narrow bars shown above the viral RNA show the positions of the reading frames for *vif*, *vpr*, *tat*, *rev*, and *nef*. The numbers are the nucleotide positions given by Ratner et al. (19).

that the RNAs containing these exons were first spliced to form transcripts for *vif* and *vpr* and then, before exiting the nucleus, underwent a subsequent splicing step which removed the *vif*- and *vpr*-coding regions, forming *rev* and *nef* mRNAs instead.

In order to more precisely quantitate the relative expression levels of *tat*, *rev*, and *nef* mRNAs, the M13 clones of the amplified fragments were plated and sequentially hybridized to the battery of oligomer probes. Nearly 80 and 20% of the clones of amplified cDNA represented *nef*- and *rev*-specific transcripts, respectively. Only 3 of 169 clones analyzed were from *tat* mRNA.

Southern blots of the amplified mRNA from macrophages infected with the BA-L strain of HIV-1 contained, in addition to bands corresponding to the main *tat*-, *rev*-, and *nef*-specific fragments noted with amplified RNA from

HTLV-III$_B$-infected H9 cells, a predominant band of approximately 320 bp. This band hybridized with a probe for the third exon (Fig. 1, exon E) of the doubly spliced viral mRNA but failed to hybridize with a probe for the middle exon. The apparent *tat*-specific band, as with the amplified infected H9 cell RNA, was relatively quite faint.

DNA sequence analyses of the amplified transcripts cloned into M13 showed that the putative *tat*, *rev*, and larger *nef* transcripts corresponded precisely to the three exon forms which constituted the predominant *tat*, *rev*, and *nef* transcripts of the infected H9 cells. In addition, fragments which did not hybridize to any of the probes for the middle exon were found to contain only the first and third exons of the doubly spliced mRNAs. Most were spliced from the major splice donor at base 287 to the major splice donor at base 7956, but some heterogeneity was evident. These cDNAs would correspond to a mRNA of approximately 1,550 bp. It is not clear whether these two exon *nef* mRNAs are formed by a single splicing step or whether they instead are formed by a third splicing step which removes the middle exon on the doubly spliced transcripts.

Hybridization of the oligonucleotide probes to plaque lifts of plated M13 clones of the amplified RNA from infected macrophages allowed quantitation of the relative expression of transcripts for *tat*, *rev*, and *nef*. As with the infected H9 cells, the great majority (77%) of these transcripts from macrophages infected with HIV-1 (BA-L) were *nef* specific. In contrast to the H9 cells, however, a majority (204 of 274 clones) of the *nef* transcripts were of the two-exon form, consistent with the intensity of the 320-bp fragment in ethidium bromide-stained gels of amplified cDNA from infected macrophages. This result contrasts with that for the cDNA from infected H9 cells, from which only 23 of 134 *nef* clones analyzed were of the two-exon form. We were unable to detect any clones of the macrophage cDNA which contained either of the small upstream exons contained in some of the *nef*- and *rev*-specific clones from the infected H9 cells.

We have shown that *nef* is by far the most abundant of the small multiply spliced transcripts of HIV-1-infected cells. This finding is independent of the strain of virus and the cell type and is seen with both chronic and short-term infections. Relatively, *tat* is expressed at extremely low levels, while *rev* is present at intermediate levels. The reason for this balance is not clear. *nef* has been reported to have a negative effect on virus replication (13, 17), but despite high levels of expression of *nef*-specific transcripts compared with those for *rev* and *tat*, virus is produced by the tested cell types at high levels. Thus, *nef* under some circumstances is relatively inefficient at inhibiting virus expression. This result, taken together with the high level of expression of *nef* in a variety of types of infection, suggests that *nef* may play another, more critical role in the viral life cycle in vivo which may be related to its reported GTP-binding activity (9).

Despite the similarities in the relative expression of *tat*, *rev*, and *nef* in H9 cells and macrophages, there are also differences. Most of the *nef* transcripts from macrophages contained only two exons, while those from the H9 cells were mostly of three exons. Also, about 10% of the *nef* and *rev* transcripts from the H9 cells contained small exons (from bases 4491 to 4542 and 4970 to 5043) derived from the regions immediately 5′ to the coding regions of *vif* and *vpr*. These transcripts with small upstream exons may be derived from *vif* and *vpr* mRNAs by a subsequent splicing event. We were unable to find any clones of macrophage cDNA containing either of these exons. This suggests that either infected macrophages express fewer *vif* and *vpr* mRNAs than infected H9 cells or they more efficiently remove these exons.

The splicing pathways of HIV mRNA are obviously quite complex. We suggest that the two-exon *nef* transcript may represent the final product of the viral splicing pathway and that all transcripts might ultimately be processed to this form if they remained in the nucleus for a sufficient time. This may not be the case, since the first splicing event could disrupt consensus donor and acceptor sequences for other splices. However, if this is true, it would suggest that viral mRNA is not processed out of the nuclei of infected macrophages as rapidly as with infected H9 cells. Aside from these differences, however, the balance of expression of *tat*-, *rev*-, and *nef*-specific transcripts seems comparable in infected macrophages and lymphocytes.

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