TRBP1 and TRBP2 are isoforms of a double-stranded RNA-binding protein that differ in their N-terminal end and were each identified by binding to human immunodeficiency virus type 1 (HIV-1) trans-activation-responsive RNA. TRBP1 and TRBP2 also bind and modulate the function of the double-stranded RNA-activated protein kinase, protein kinase R. Both proteins increase long terminal repeat expression in human and murine cells, and their gene has been mapped to human chromosome 12. We have isolated and characterized the complete tarbp2 gene (5493 bp) coding for the two TRBP proteins. Two adjacent promoters initiate transcription of alternative first exons for TRBP1 and TRBP2 mRNAs that are spliced onto common downstream exons. TRBP2 transcription and translation start sites are localized within the first intron of TRBP1. TRBP promoters are TATA-less but have CCAAT boxes, a CpG island, and several potential binding sites for transcriptional factors. Promoter deletion analysis identified two regions from position −1397 to −330 for TRBP1 and from position −330 to +38 for TRBP2 that are important for promoter function. TRBP2 promoter activity was expressed at a higher level compared with TRBP1 promoter. In addition, a specific down-regulation of TRBP1 and TRBP2 promoter activity was identified in human astrocytic cell line U251MG compared with HeLa cells. This minimal TRBP promoter activity may account for minimal HIV-1 replication in astrocytes.

TRBP (trans-activation-responsive RNA-binding protein), a cellular protein that binds HIV-1 TAR RNA and increases viral expression from the long terminal repeat (LTR) (1). Two forms of the protein, TRBP1 (or TRBP) and TRBP2, coexist in the cell and are encoded by two different mRNAs that differ by their 5'-untranslated region (5'-UTR). TRBP2 protein is 21 amino acids longer than TRBP1 (1–3). TRBP proteins belong to the family of double-stranded RNA-binding proteins (4–7). TRBP1 and TRBP2 have two double-stranded RNA-binding domains and a C-terminal basic region (8), but only one double-stranded RNA-binding domain is functional with regard to TAR binding because of the presence of a KR-helix motif. This 15-amino acid peptide motif is the TRBP minimum TAR RNA-binding motif that binds preferentially to GC-rich oligoribonucleotides and destabilizes the TAR RNA structure (5, 9, 10).

In the context of HIV, TRBP increases the basal expression of the promoter and the Tat-activated level of the HIV-1 LTR in human and murine cells (1, 3). In vivo, TRBP binds to TAR RNA (5) as well as to the double-stranded RNA-induced, interferon-regulated protein kinase PKR (11–13). TRBP blocks the inhibitory effects of PKR on translation (14), on HIV LTR expression (8, 15), and on HIV replication (13). TRBP-PKR dimerization sites are located in each double-stranded RNA-binding domain in TRBP. The interaction domains between the two proteins reverse PKR inhibition on yeast growth and on HIV-LTR activity (8). All of the available data suggest that TRBP facilitates viral replication by two mechanisms: direct activation of the LTR through TAR binding and inhibition of the host antiviral mechanisms through increased translation.

During HIV infection, the central nervous system is an important viral reservoir that contributes to viral persistence (16). HIV invades the brain and infects astrocytes and microglia early in the course of the disease. Astrocyte infection is remarkable for the low production of new virus (17, 18). Reports indicate that this dormant HIV infection in primary human astrocytes and astrocytoma cell lines is due to either a block in Rev function and/or inefficient translation of HIV structural proteins (19–21). Report differences are not currently explained. A recent observation indicates that HIV replication can be restored in astrocytes by coexpression of TRBP or a catalytically inactive PKR mutant. Furthermore, astrocytes express a very low level of endogenous TRBP, which may
explain the restoration of normal translational function by the protein. These results suggest that a heightened responsiveness of the interferon-induced PKR pathway in astrocytes makes these cells refractory to HIV-1 replication. They also point out a key role for TRBP in HIV replication. Although these results do not explain the restoration of a deficient Rev function observed by others in astrocytes (19, 20), a role of TRBP in this process is not excluded because TRBP has been shown to bind Rev-responsive element RNA (14).

Genetic analysis showed that TRBP is encoded by the tarbp2 gene mapped to human chromosome 12 and mouse chromosome 15 (22, 23). Pseudogenes have been located on human chromosome 8 and mouse chromosome 6 and 7 (3, 22). Despite an extensive search for the human tarbp2 gene in genomic libraries prepared in λ phage, no clones were isolated. Here, we describe the characterization of the tarbp2 gene in a YAC clone and the analysis of its complete sequence cloned from genomic DNA. We report the identification of two promoter regions that specifically transcribe TRBP1 and TRBP2 mRNAs and show the expression of the human tarbp2 gene in the same region of chromosome 12 were obtained (25). YAC/H11032 (MD19, 5/gt/1032 France) after PCR screening with oligonucleotides from TRBP cDNA point out a key role for TRBP in HIV replication.2 Although these results do not explain the restoration of a deficient Rev function, they highlight the importance of TRBP in HIV replication.

2 J. Thorne, C. Ong, A. Gatignol, and D. F. Purcell, manuscript in preparation.

**EXPERIMENTAL PROCEDURES**

**Yeast and Human DNA Extraction—**YAC clones (24) were obtained from D. Le Paslier (Center d’Etude du Polymorphisme Humain, Paris, France) after PCR screening with oligonucleotides from TRBP cDNA (MD19, 5-CCACCGCAAAGATTCAACA-3′ and MD20, 5-CCGC-GGATTCCGGTCCG-3′). One positive clone and nine other candidates in the same region of chromosome 12 were obtained (25). YAC clones were grown on selective AHC medium at 30 °C for 3 days. Red colonies were isolated, and the yeast DNA was extracted based on previous protocols (26). Human genomic DNA was isolated from HeLa cell lines as previously described (27). Human genomic DNA from placenta was purchased from CLONTECH.

**Genomic DNA Cloning—**PCR amplifications were performed in 100 μl of reaction mixture containing 200 ng of genomic DNA or 10 ng of pBS-TRBP2 as control (2), 250 ng of each primer, 2.5 units of Taq DNA Polymerase (Invitrogen), 1.5 mM MgCl2, 0.2 mM dNTP, and 1× PCR buffer (Invitrogen). The conditions for the amplifications were 95 °C for 5 min; 40 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and incubation at 72 °C for 5 min. The reactions were performed in a thermocycler (Biometra) and run on a 1.5% agarose gel for size determination. TRBP gene PCR products were excised from agarose gels and eluted with a QiAquick kit (Stratagene) and inserted into pPCR-Script™ Amp SRI (+) plasmid cloning vector (Stratagene).

**Plasmid Constructions—**The pGL4 expression vector system (Promega), containing firefly luciferase as a reporter gene, was used to measure the promoter activity of cloned genomic DNA fragments from the 5′ region of the TRBP gene. Genomic DNA fragments were obtained by PCR as previously described for the genomic PCR subcloning. Primers derived from the sense and antisense strands of the TRBP promoter region were synthesized (Invitrogen) with additional terminal adapter sequences for KpnI, SacI, or NotI restriction sites (Table I). PCR products were cloned into the multiple cloning site of pGL4-basic vector, which does not contain any promoter or enhancer element. Correct insertion and sequence was confirmed by sequencing. pGL4-control vector that contains the SV40 promoter and enhancer sequences was used as control (Promega). Similarly, plTR-Luc was constructed by inserting the HIV-1 LTR into pGL4-basic vector (3). DNA sequencing of both strands of clones was performed on an automated DNA sequencer ABI373A (Applied Biosystems) using the Bigdye terminator chemistry.

**Southern Blot Analysis—**10 μg of YAC or 20 μg of human genomic DNA were digested with EcoRI endonuclease, then electrophoresed on a 0.7% agarose gel, and transferred to Nitran plus nylon membranes (Schleicher & Schuell). The filter was incubated in hybridizing solution (20 mM phosphate buffer, pH 7.5, containing 5× SSC, 7% SDS, 10× Denhardt’s solution, and 1% salmon sperm DNA) with 9× 106 cpm of 32P-labeled TRBP cDNA probe. After overnight hybridization at 42 °C, the membrane was washed twice in 2× SSC, 2% SDS at 20 °C, washed once in 0.1× SSC, 0.1% SDS at 50 °C for 15 min, and exposed 24 h to x-ray film. The membrane was rehybridized with a 32P-labeled pGL4-control cDNA probe.

**Primer Extension Analysis—**Total RNA was isolated from Jurkat cell lines using the TriPure isolation reagent (Roche Molecular Biochemicals). TRBP-specific primers were 5′-radialolabeled using T4 Polynucleotide kinase (Invitrogen) and [γ-32P]ATP (Amersham Biosciences, Inc.). Primer sequences were: 5′-CGGTTITGGAAGTGCTGATCAGGCGTCT-3′ (A primer), 5′-CTGCAAGAAGCCTGTGATCCAGGCTG-3′ (B primer), 5′-CTGCAAGAAGCCTGTGATCCAGGCTG-3′ (C primer), and 5′-GTTATACACATAGGCGGTGCG-3′ (D primer). [γ-32P]ATP-specific primer (125 ng) and total RNA (20 μg) were ethanol coprecipitated, and the dried pellet was resuspended in 30 μl of 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 μM NaCl, 80% formamide, denatured at 85 °C for 10 min, and incubated at room temperature for 30 min and overnight at 30 °C. The reaction mixtures were ethanol-precipitated. Dried pellet was used to synthesize the single strand cDNA. The reaction mix containing 30 units of RNasin (Amersham Biosciences, Inc.), 1 μl of dNTP, 10 μl dithiothreitol, 300 units of Superscript II (Invitrogen) in a 25-μl volume was incubated at 42 °C for 2 h. 10 units of RNase A were added, and the samples were incubated at 42 °C for 30 min prior to phenol-chloroform extraction and ethanol precipitation. Dried samples were resuspended in 40 μl of water. 4 μl were mixed with 97.5% formamide-dye mix and run on 6% acrylamide, 7 M urea gels, which was dried and exposed for autoradiography. To determine the cDNA size, a sequencing reaction was performed with a T7 DNA polymerase sequencing kit (Amersham Biosciences, Inc.) according to the manufacturer’s protocol and run on the same gel.

**Transient Transfections and Luciferase/Renilla Assays—**HeLa and glioblastoma/astrocytoma cell line U251MG cells were grown in Dulbecco’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone) in 12-well plates to reach 60–80% confluence at the time of transfection. Each transfection involved cotransfection with 5 μg of pGL4-basic containing different TRBP fragments or pGL4-control or plTR-Luc vector and 1 μg of control plasmid in which the Renilla gene is expressed from the thymidine kinase promoter (pPL-TK; Promega). DNA was transfected by calcium phosphate coprecipitation.
phosphate precipitation according to the manufacturer's protocol (Stratagene). 18 h after the transfection, the cells were rinsed with serum-free Dulbecco's minimal essential medium and incubated for 24 h with Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum. The cells were washed three times with phosphate-buffered saline and lysed in 150 μl of lysis buffer. The luciferase/ Renilla coexpression was measured with 20 μl of cell lysates by luminescence method according to the manufacturer's protocol (Promega). Each transfection was performed in triplicate and was repeated three times. The luciferase activity of each sample was normalized to the Renilla activity.

**RT-PCR and Semi-quantitative RT-PCR Analysis—**Total RNA was isolated from HeLa, Jurkat, and U251MG cell lines using the tripure isolation reagent and treated with DNase (Roche Molecular Biochemicals). TRBP cDNA was amplified from 5 μg of total RNA using 5 pmol of TRBP1/2 antisense- (5'-GTGCCTTGCTGGGTGGGC-3') or GAPDH antisense-specific primer (5'-CCAAAGTTGTCATGGATGACC-3') in a 5-μl reaction containing 30 units of RNasin (Amersham Biosciences, Inc.), 1 mM dNTP, 10 mM dithiothreitol, and 300 units of Superscript II (Invitrogen). Incubation was performed at 42 °C for 2 min, 55 °C for 1 min, and 72 °C for 2 min; and a 5-min incubation at 72 °C. PCR amplifications were performed in a 200-μl reaction mixture containing 250 ng of each GAPDH (5'-CCTCTATTGCCTCAACTACAT-3') or TRBP primer (TRBP1 primer -302/-286: 5'-CCAGCTGGGACACAGTCAGG-3', TRBP2 primer +38/+56: 5'-GGGGACTCCATATCCCAG-3'), 2.5 units of Taq DNA polymerase (Invitrogen), 1.5 mM MgCl2, 0.2 mM dNTP, and 1× Taq buffer (Invitrogen). Antisense primers were described above for reverse reaction. To verify the 5' extremity of TRBP and TRBP2, three different sense primers were used: TRBP1, -276/-258 5'-GCTCTTGGTGTTCTGTAGT-3', TRBP2, +38/+56 5'-GGGGACTCCATATCCCAG-3', 2.5 units of Taq DNA polymerase (Invitrogen), 1.5 mM MgCl2, 0.2 mM dNTP, and 1× Taq buffer (Invitrogen). Antisense primers were described above for reverse reaction. To verify the 5' extremity of TRBP and TRBP2, three different sense primers were used: TRBP1, -276/-258 5'-GCTCTTGGTGTTCTGTAGT-3', TRBP2, +38/+56 5'-GGGGACTCCATATCCCAG-3', 2.5 units of Taq DNA polymerase (Invitrogen), 1.5 mM MgCl2, 0.2 mM dNTP, and 1× Taq buffer (Invitrogen). Antisense primers were described above for reverse reaction.

**RESULTS**

**Isolation of Genomic TRBP Clones—**Several approaches have been used to clone genomic DNA before the completion of the human genome project. They include screening from YACs or bacterial artificial chromosomes and PCR methods from genomic DNA or libraries. We have selected primers separated by 57 bp in TRBP cDNA that give rise to a 400-bp band after genomic DNA or libraries. We have selected primers separated by 57 bp in TRBP cDNA that give rise to a 400-bp band after genomic DNA or libraries. We have selected primers separated by 57 bp in TRBP cDNA that give rise to a 400-bp band after genomic DNA or libraries. We have selected primers separated by 57 bp in TRBP cDNA that give rise to a 400-bp band after genomic DNA.

**Fig. 1.** Characterization of the tarbp2 gene in human genomic DNA and in YAC 791E7. A, PCR analysis of pBS-SK plasmid (lane 2), pBS-TRBP plasmid (lane 3), and genomic DNA from HeLa cells (lane 4) using TRBP primers MD19 and MD20. Lane 1 (M) shows molecular mass markers. B, ethidium bromide staining (top) and Southern blot hybridization (bottom) using 10 or 20 μg of EcoRI-digested YAC or genomic DNA, respectively, probed with 32P-labeled TRBP cDNA (lanes 2–12). Lane 1 (M) shows molecular mass markers. C, PCR analysis using DNA from human placenta (lane 1) and from YAC 791E7 (lane 2) performed with MD19 and MD20 primers.

| Lane | Description |
|------|-------------|
| 1    | Molecular mass markers |
| 2    | pBS-SK plasmid |
| 3    | pBS-TRBP plasmid |
| 4    | Genomic DNA from HeLa cells |
| M    | Molecular mass markers |

The absence of the tarbp2 gene in other YACs mapped in 12q12-q13 supports this hypothesis (Fig. 1B). This YAC has probably the entire gene sequence because the 3.4-, 2.1-, and 1.5-kb EcoRI bands have been attributed to the gene (3, 22).
FIG. 2. Structure of the tarbp2 gene and primer extension analysis of TRBP1 and TRBP2 mRNAs. A, schematic representation of tarbp2 gene deduced from GenBank sequence AC023509 aligned with TRBP1 and TRBP2 cDNAs. The exons (boxes) and introns (line) of the human tarbp2 gene are represented. The CpG islands contained in the promoter region and primers (arrows A–D) used for primer extension reaction are indicated. B, primer extension analysis. 20 μg of total RNA from Jurkat cell line were hybridized with 125 ng of TRBP specific primers labeled with [γ-32P]ATP and polynucleotide kinase. The extension reaction was performed as described under "Experimental Procedures." The products were analyzed on a 6% acrylamide, 7 m urea gel. The DNA sequence ladder served as size markers for the extension products. The arrows indicate the extension products corresponding to TRBP1 (left panel) and TRBP2 (right panel) mRNAs. The primers used for each reaction are indicated. C, predicted secondary structure of the first 99 and 183 nucleotides upstream of the ATG of TRBP1 and TRBP2 mRNA, respectively. Folding was performed using mfold server 3.1 (30,31) and gave ΔG free energy values of −31.1 and −92.4 kcal/mol, respectively.
PCR analysis of YAC 791E7 and the human genomic DNA revealed the presence of at least eight introns that were identical to those identified after the release from large scale sequencing of the human genome. This release revealed the presence of sequences identical to TRBP1 and TRBP2 cDNAs (GenBank accession number AC023509). From these unaligned sequences, a single tarbp2 gene was found in a 10,478-bp fragment, and the sizes of the hybridizing bands as aligned sequences, a single tarbp2 promoter might be located in the proximity of the CpG island and upstream from the cDNA start sites.

Two Promoters in the Human tarbp2 Gene

Mapping the TRBP1 and TRBP2 Transcription Start Sites by Primer Extension Analysis—5′-Terminal ends of TRBP1 and TRBP2 mRNAs have been first determined from cDNA sequences (1, 2) and extended after PCR cloning of additional sequences from a cDNA library (3). Because libraries do not always reflect the exact 5′ ends, we performed primer extension analysis using Jurkat total RNA and four different primers to determine the precise 5′ extremity of TRBP1 and TRBP2. Primers were chosen to be either common for both mRNAs (primer A and B in exon 3) or specific for TRBP2 (primers C and D in exon 2; Fig. 2A). Primers A and B located in exon 3 gave rise to major bands at 192 and 140 bp, respectively (Fig. 2B). The smaller band observed with primer A was not reproducible and is probably due to an unspecified step of reverse transcriptase. The product size difference corresponds to the primer positions in exon 3 and therefore reveals the same mRNA with an expected size difference. Primer extension experiments with primers C and D revealed major products at 144 and 141 bp (Fig. 2B), also indicating that they initiated from a single mRNA. Surprisingly, primers A and B did not give rise to the higher molecular mass bands expected from an extension through exon 2. One explanation for these size products is that primers A and B only extend from exon 3 to exon 1 and reveal TRBP1 mRNA. Primers C and D extend from exon 2 and reveal TRBP2 mRNA with an extended 5′ end terminus. To definitively prove how these mRNAs are generated and to confirm their transcriptional start sites, RT-PCR reactions were performed with different sense primers located in the 5′ region of TRBP1 and TRBP2 (Fig. 3A). For each mRNA, PCR reactions were performed with two sense primers located downstream of the transcription start site (lanes 1–4 and 7–10 for TRBP1 and TRBP2, respectively). The absence of PCR product with sense primers located upstream of each transcription start site (lanes 6 and 12) confirmed the primer extension results. From the sequence of all RT-PCR products, we concluded that TRBP1 mRNA contains exons 1 and 3 and that TRBP2 mRNA contains exons 2 and 3. These data also showed that TRBP1 mRNA is 36 nucleotides longer than previously reported and that TRBP2 mRNA can be extended by an additional 92 nucleotides. To understand why primers A and B did not extend TRBP2 mRNA and to verify whether the 5′ end of the mRNAs can fold into the secondary structure previously observed (3), a folding analysis of each 5′ end mRNA was performed using the RNA mfold server version 3.1 (30, 31). TRBP1 and TRBP2 5′ extremities folded into stable stem-loop structures as illustrated in Fig. 2C. TRBP2 5′ end predicted structure (−92.4 kcal/mol) was more stable than the corresponding end in TRBP1 (−31.1 kcal/mol), because of the relatively greater GC content and the longer predicted stem structures. This new structure for TRBP2 mRNA extends the stem-loop previously described (3). It is likely that this GC-rich structure prevents the extension of primers A and B by reverse transcriptase when they hybridize to TRBP2 mRNA. Therefore, we only observed the extension from TRBP1 mRNA with these primers. Hybridization of primers C and D that specifically bind within this predicted structure might destabilize TRBP2 stem-loop and facilitate reverse transcription. Overall, these experiments show that TRBP1 and TRBP2 are produced from independent transcription start sites that generate novel pre-mRNAs that are alternatively spliced into the common splice acceptor site of exon 3 (Fig. 3B).

Genomic Organization Shows an Alternative First Exon for TRBP1 and TRBP2 mRNAs and a Constitutive Splicing of Downstream Exons—To determine the position and the features of tarbp2 promoter region, the overall gene organization from the primer extension data and from sequence comparison with the TRBP cDNAs were analyzed. The 10 exons of tarbp2 gene ranged from 72 to 433 bp and introns from 139 to 841 bp. The exon/intron boundaries are all identical to the gt/ag consensus sequence, and a pyrimidine-rich region is present in the introns upstream of the 3′ acceptor sites (Table II) (32, 33). TRBP1 and TRBP2 are each encoded by nine exons, and their respective first exons are exon 1 and 2 (Fig. 3B). Whereas exon 1 expresses the 5′-UTR of TRBP1 mRNA, exon 2 is only present in TRBP2 mRNA and contains the translation initiation site of TRBP2. For TRBP1 mRNA synthesis, exon 2 is excised with its first intron. Exon 3 contains the TRBP1 translation start site and TRBP2 coding region (Fig. 3C). The splicings between exon 1 and exon 3 (for TRBP1) and between exon 2 and exon 3 (for TRBP2) occur through the same 3′ acceptor site in intron II (Table II). The open reading frame is conserved between these two mRNAs, and TRBP2 protein has 21 additional N-terminal amino acids compared with TRBP1 as previously described (2, 3). Comparison of this genomic region with cDNA sequences in expressed sequence tag databases showed several mRNAs identical to TRBP1 and TRBP2 but also additional TRBP forms produced by alternative splicing of exons 3–10. This result suggests the presence of other TRBP isoforms in different cell types or tissues.

Sequences Upstream of TRBP1 and TRBP2 Start Sites Show No TATA Box—To characterize the promoter features of tarbp2 gene, the sequences upstream of the TRBP1 and TRBP2 mRNA start sites were examined, and the TRBP2 transcription start site was chosen as +1 position. No TATA box could be identified in either the exon 1 or exon 2 upstream regions and the sequence around the start sites do not have an initiator-like sequence (34). The genomic sequence was analyzed for potential transcription factor-binding sites using a transcription factor database (MatInspector) (35, 36). Three CAAT boxes and several potential CAAT enhancer-binding protein β-binding sites were located upstream of the TRBP1 transcription start site. Computer sequence predictions also identified binding sites for Sp1, AP1, AP2, AP4, NFAT, MZF-1, RFX1, NFY, CEBP, and GATA transcription factors (Fig. 4). Some of these binding sites are within the CpG island previously mentioned, located between positions −588 and −329. About 50% of all mammalian genes possess a CpG island near their transcrip-
tion start site (29), and GC-rich sequences are often found in TATA-less promoters (37, 38). The 5′-flanking region also contains two interferon γ-activated sequence elements (TTCN2−6GAA), suggesting that TRBP expression might be regulated by interferons. Interestingly, there are four sex-determining region Y sites (AACAAT) that may bind the corresponding testis determining factor (39). Because the murine TRBP homologue, PRBP (protamine 1 RNA-binding protein), has been shown to

**Fig. 3.** TRBP1 and TRBP2 mRNAs are produced by a different splicing mechanism. A, RT-PCR analysis. 5 μg of total Jurkat RNA were reverse transcribed and subjected to PCR amplification with primers spanning the 5′ extremity of TRBP1 (lanes 1 and 2, primer −276/+258; lanes 3 and 4, primer −302/+286; lanes 5 and 6, primer −462/+444) and TRBP2 (lanes 7 and 8, primer +70/+87; lanes 9 and 10, primer +37/+55; lanes 11 and 12, primer −127/−108). Negative controls were performed by PCR amplification without reverse transcriptase (lanes 1, 3, 5, 7, 9, and 11). No PCR products were observed with sense primers located upstream of transcription start site (lanes 6 and 12). Molecular mass markers are indicated (left lane). B, schematic representation of the splicing mechanism that generates TRBP1 and TRBP2 mRNAs from tarbp2 gene. The exons (boxes), the introns (line), and the splicing events in human tarbp2 gene are indicated. The arrows represent the two transcription start sites. Exons 1–3 involved in the alternative transcription start site and splicing between TRBP1 and TRBP2 are differentially drawn. TRBP1 mRNA is obtained when the transcription starts in exon 1. TRBP2 mRNA is obtained when the transcription starts in exon 2. The two splicing events use the same acceptor site at the exon 3. C, mRNA organization obtained after splicing of the tarbp2 gene. TRBP1 mRNA is produced from exon 1 and exons 3–10. TRBP1 translation start site (AUG) is located at the 5′ extremity of exon 3. TRBP2 mRNA is produced from exons 2–10. TRBP2 translation start site is located at the 3′ extremity of exon 2. The open reading frame is conserved between TRBP1 and TRBP2. nts, nucleotides.
be involved in spermatogenesis, this factor might regulate human TRBP expression in testis (40).

TRBP1 and TRBP2 Are Encoded from Two Different Promoters—The presence of two different start sites for TRBP1 and TRBP2 mRNAs separated by 377 nucleotides, and the absence of a TATA box indicated no obvious location for tarbp2 promoter. We therefore cloned different fragments upstream of a luciferase reporter gene to determine promoter activity. Constructs were designed to test the 5′-flanking region of TRBP1, TRBP2, or both as well as the role of the CpG island (Fig. 5 A). The region upstream of TRBP1 start site was first examined by transfection in HeLa cells (Fig. 5 B). The largest fragment spanning from positions −1397 to −227 including TRBP1 mRNA start site led to the highest luciferase activity and was set as 100%. Successive deletions in the 5′ end region up to −1255, −855, −587, and −461 showed 59, 68, 29, and 43%, respectively, indicating the presence of a functional promoter. The region including positions −330 to −227 showed 6% activity and was considered to be nonfunctional. The region spanning from −461 showed 43% promoter activity and likely represents the minimal promoter. Other regions have various modulating effects, particularly the sequence between −587 and −461 has inhibitory effects, whereas other upstream regions have mainly enhancer functions. Therefore, the overall promoter enhancer region for TRBP1 is located between positions −1397 and −330 and was called TRBP1 promoter (Fig. 5 B).

The previous constructs include TRBP1 but not TRBP2 mRNA start site, and it was not clear whether this promoter region can direct transcription for both mRNAs. To elucidate this mechanism, the sequence that includes TRBP2 mRNA start site (−226 to +157) was added to the constructs (−1397 to −227 and −330 to −227) that gave the highest and the lowest activity, respectively. The largest fragment (−1397 to +157) had a 3-fold increased activity compared with the previous corresponding sequence (−1397 to −227), suggesting the presence of either an enhancer element or a second promoter in region −227 to +157 (Fig. 6, compare lanes 4 and 5). The results for the fragment spanning from −330 to +157 showed high promoter activity (Fig. 5 C), whereas the corresponding sequence upstream TRBP1 start site (−330 to −227) had no activity (Fig. 5 B). This result indicates the presence of a second promoter in this region, specific for TRBP2 mRNA expression. This region was designated the TRBP2 promoter, and its activity was set to 100% for further comparison. Successive deletions showed a progressive decrease of TRBP2 promoter activity; at +38 nucleotide only 15% activity remained. Thus, the TRBP2 promoter is located in the region between positions −330 and +38. The 15% activity of the +38/+157 fragment indicates that the region downstream of the +1 site can still promote minor transcription start sites. This activity increases the promoter function because the removal of sequences from +100 to +157 decreased the −244 TRBP2 promoter activity (Fig. 5 C, bottom two rows).

TRBP1 and TRBP2 Promoters Have a Similar Pattern of Activity in Astrocytic U251MG and HeLa Cell Lines—Because TRBP low expression has been suggested as a cause of low HIV replication in astrocytes,4 we investigated whether this lack of protein was from transcriptional origin. To determine the transcriptional activity of the TRBP promoters in astrocytes, the deletion constructs described above were expressed in U251MG cells (Fig. 5, D and E). As in Fig. 5 (B and C), constructs (TRBP1, −1397 to −227, and TRBP2, −330 to +157) were set up to 100% activity. The pattern of expression of TRBP1 and TRBP2 promoters was similar in U251MG compared with HeLa cells. Sequences located between −1255 and −855 have a higher repressor activity in U251MG cells (48% inhibition) compared with HeLa cells (9% inhibition). The RFX1-binding site present in position −980 (Fig. 4) has previously been shown to mediate repression functions and might be involved in this process (41). Constructs with TRBP2 promoter had a similar profile in HeLa and U251MG cell lines (Fig. 5, C and E), although a minor difference can be observed for sequences between −330 and −244. This region has an activating function in HeLa cells and an inhibiting activity in U251MG cells. These results suggest that TRBP1 promoter region has a specific silencer region that is more active in astrocytes, whereas TRBP2 promoter regulation shows only small pattern differences between U251MG and HeLa cells.

TRBP1 and TRBP2 Promoters Are Weakly Expressed in Astrocytic U251MG Cell Line—Astrocytes have a low level of translation of the HIV proteins Gag, Pol, and Env during HIV infection (21) and also express low levels of TRBP. We therefore sought to determine whether these cell lines have a general low translational activity or whether a specific repression mechanism acts on TRBP1 or TRBP2 promoter. We therefore compared TRBP promoter expression to the expression of HIV-1 LTR and SV40 promoters in HeLa and U251MG cells. The expression of TRBP1 promoter (Fig. 6, lanes 5–10), TRBP2 promoter (lanes 11–14), or both (lane 4) showed a dramatic decreased activity in U251MG compared with HeLa cells. TRBP1 (lane 5) and TRBP2 (lane 11) promoters were expressed at 9.4- and 5-fold lower levels in the astrocytic cell line compared with HeLa, whereas the TRBP1 and TRBP2 promoters together (lane 4) showed a 5-fold decrease. As a control, the SV40 (lane 2) and the HIV-1 (lane 3) promoters showed similar activities in both cell lines. A direct comparison between

Table II

| Exon Number | Size (bp) | Splice donor and acceptor | 3' acceptor | Intron Number | Size (bp) |
|-------------|----------|--------------------------|------------|--------------|----------|
| 1           | 85       | CCCAA/gttgag............. | ...ggacccctccccagt/TATAG | 1            | 287      |
| 2           | 233      | CCTAG/gttgac............. | ...ggacccctccccagt/TATAG | 2            | 552      |
| 3           | 169      | CACTG/gttgag............. | ...gtcccccctctctcag/GTCAG | 3            | 841      |
| 4           | 102      | AGGAG/gttgag............. | ...ctttcccccccgtgtag/TCTTT | 4            | 582      |
| 5           | 95       | ACCAG/gttatct.........   |...gtctctccccctcag/GACCC | 5            | 587      |
| 6           | 72       | TGCAG/gttgctc.......... |...gttctctccccccag/GAGCT | 6            | 227      |
| 7           | 117      | GATTG/gttgc............. |...ctttcccccccgtgtag/GAGCT | 7            | 318      |
| 8           | 127      | CATTG/gttgag........... |...gtctctccccctcag/GGTG | 8            | 386      |
| 9           | 201      | TATTG/gttgag........... |...gtctctccccctcag/GAGAG | IX           | 139      |
Two Promoters in the Human tarbp2 Gene

TRBP1 and TRBP2 promoter activities indicate that TRBP2 promoter (lane 11) is 2 and 3.6 times more active than TRBP1 promoter (lane 5) in HeLa and U251MG cells, respectively. HeLa and U251MG transfection efficiencies have been verified to be identical as measured by green fluorescent protein expression (data not shown), and therefore the luciferase activity reflects the overall promoter expression. These results indicate that the 5'-flanking region and the first intron of the tarbp2 gene are different cDNAs and with a small and large arrow, respectively. Computer-identified consensus sequences for transcription factors are underlined and identified below the sequence. CCAAT boxes are in italic and underlined. The CpG island (–588 to –329) is indicated with outlined letters (GenBank™ accession number AF281068).

Endogenous TRBP1 and TRBP2 mRNA Expression Is Correlated with TRBP1 and TRBP2 Promoter Activities—To confirm the differential activity between TRBP1 and TRBP2 promoters inside the cell, we evaluated the endogenous level of TRBP1 and TRBP2 mRNAs by semiquantitative RT-PCR analysis (Fig. 7). TRBP1- and TRBP2-specific primers were chosen to amplify specifically TRBP1 and TRBP2 mRNAs, respectively. TRBP1 primer was located in exon 1, which is only present in TRBP1 mRNA. TRBP2 primer was located in exon 2, which is spliced when TRBP1 mRNA is formed. Reverse transcription was initiated in exon 3, common for both mRNAs. The results confirm that endogenous TRBP1 mRNA is more weakly expressed than TRBP2 mRNA in HeLa (lanes 1 and 4), Jurkat (lanes 2 and 5), and U251MG cell lines (lanes 3 and 6). Both mRNAs were less expressed in U251MG than in HeLa or Jurkat cells. These results prove the specific down-regulation of TRBP1 and TRBP2 expression in the astrocytic cell line U251MG compared with HeLa or Jurkat cells. Both promoters and mRNAs are less expressed in astrocytic cells compared with HeLa cells.

Previous attempts to clone the tarbp2 gene led to the isolation of its pseudogene (3, 22) and to the selection of a specific YAC that was positive in a large scale PCR screening procedure (Fig. 1). YAC 791E7 mapped to human chromosome 12p11-p12, which is compatible with the previous mapping (22) and likely contains the entire tarbp2 gene. However, the comparison with other YACs in the same region and sequence-tagged site mapping data indicated that it has recombined and inserted regions from 12q12-q13 in its sequence. The release of a chromosome 12 sequence from large scale sequencing in February 2000 oriented our studies toward the analysis of the gene organization and the cloning of the promoter region from genomic DNA. tarbp2, located on human chromosome 12q12-q13, definitively indicated that TRBP1 and TRBP2 are different cDNAs and that their specific 5' ends are found in different locations.
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Fig. 5. Analysis of promoter activity of tarbp2 5′-flanking region in HeLa and astrocytoma U251MG cells. A, schematic drawing of TRBP CpG island and exons 1–3. TRBP1 and TRBP2 transcriptional and translational start sites are indicated. TRBP1 and TRBP2 5′-untranslated regions are indicated with black boxes, and the coding regions are represented with hatched boxes. B and D, promoter activity of the region upstream of TRBP1 transcription start site in HeLa cells (B) and in U251MG cells (D). Promoter regions are represented on the left. Relative luciferase activity is represented on the graph (right) and normalized to Renilla expression. 100% activity corresponds to the expression of the largest promoter region. C and E, promoter activity of the region upstream of TRBP2 transcription start site in HeLa cells (C) and in U251MG cells (E). Promoter representations and graphs are as in B and D. 100% activity corresponds to the expression of the longest promoter region in the absence of TRBP1 5′-flanking region. Each value represents the average of three independent experiments performed in triplicate (± S.E.).

tarbp2 contains 10 exons, and each TRBP mRNA contains the sequence of nine exons. TRBP1 and TRBP2 are produced from separated transcription initiation sites that generate novel first exons in the pre-mRNAs that are spliced into the common downstream exons (Fig. 3). Exon 1 starts with the 5′-UTR of TRBP1 mRNA, whereas exon 2 starts with the 5′-UTR of TRBP2 mRNA and contains its translation initiation site. Exon 3 contains the TRBP1 translation initiation site and TRBP2 coding region (Fig. 3). The open reading frame is conserved, and TRBP2 protein contains 21 additional N-terminal amino acids compared with TRBP1. We have previously shown that these two proteins play a similar role in the activation of HIV-1 LTR expression (3), but the additional amino acids may bring a specific function in the noninfected cells. Interestingly, two isoforms of another RNA-binding protein, the La autoantigen, are produced by a mechanism similar to TRBP (e.g. an exchange of the exon 1 and an alternative promoter site within the first intron) (42). An alternative splicing mechanism has been described to produce four human Stau transcripts that change in their 5′-UTR extremities (43).

TRBP1 and TRBP2 proteins are double-stranded RNA-binding proteins with clearly defined double-stranded RNA-binding domains and a KR-helix motif that confers a strong affinity for dsGC-rich RNA to the proteins (5, 9, 10). We have previously shown that TRBP2 5′ region contains a GC-rich sequence that can fold into a stable stem-loop RNA structure (3). The primer extension studies shown here combined with RT-PCR and sequence analysis show that this structure is indeed longer and more highly structured for TRBP2 mRNA and that a TAR-like structure exists for TRBP1 mRNA (Fig. 2C). This folding is currently obtained only by RNA mfold analysis, and experiments to confirm this structure will be performed by enzymatic and chemical cleavage of the RNA. The prediction of very stable structures is generally very similar to those verified experimentally (44), but enzymatic and chemical probing often reveal local specific structures (45). Both structures are GC-rich, and it is likely that TRBPs will bind their own mRNAs and exert a regulatory function through this interaction. The differential structure between TRBP1 and TRBP2 5′ ends might be involved in the regulation of TRBP protein expression by auto- or cross-regulation. The potential interaction of TRBPs or other factors with these structures might influence the stability, the cellular localization, or the translation ability of the respective mRNAs. These activities might modulate their own expression in different cell lines or tissues as well as in response to viral infection or cellular stimuli.

The presence of two different start sites for TRBP1 and TRBP2 mRNAs raised the question of the presence of one or two promoters for TRBP and the mechanism of its regulation. The region upstream of TRBP1 and TRBP2 mRNA start sites show features of housekeeping-like promoters with no TATA box, but GC boxes, a CpG island, and CCAAT boxes. The analysis of the promoting activity of various fragments in this region showed the presence of two different promoters: one upstream of TRBP1 start site and one upstream of TRBP2 start site within intron I. The activity of these promoter regions suggests different positive and negative regulations (Fig. 5, B and C). Comparison of the strength of each promoter indicates that TRBP2 promoter is twice stronger than TRBP1 and that the presence of both has an additive effect on reporter gene expression (Fig. 6, lanes 4, 5, and 11). This result suggests that the production of the two mRNAs has an independent regulation, and semi-quantitative RT-PCR confirms that TRBP2 mRNA is produced with higher efficiency than TRBP1.

HIV replication depends on a large number of steps that are dependent on viral and cellular factors. Important factors can
be identified by using models that do not replicate the virus efficiently. Astrocytoma/glioblastoma cells have been shown to replicate HIV poorly, and the defect has been attributed to a lack of Rev function and a poor translation efficiency (20, 21). Recent data show that HIV replication can be restored by overexpression of TRBP and that TRBP protein is present in very low amount in the astrocytic cell line U251MG and in primary fetal astrocytes. By RT-PCR we show a lower TRBP mRNA level in U251MG than in HeLa and Jurkat cells, which suggests a transcriptional regulation or a defect in mRNA stability (Fig. 7). These results lead us to investigate whether TRBP was regulated at the transcriptional level in this cell line. When normalized to a 100% maximum activity, TRBP1 and TRBP2 promoters have a similar pattern of expression, except for a promoter fragment (−1255 to −855) that has a stronger silencer activity in astrocytes compared with HeLa cells (Fig. 5, B and D). Either a specific repressor or the lack of an activator might be responsible for this observation. A more striking observation was that all of the constructs that have TRBP1, TRBP2, or both promoters were expressed between 2 and 9.4 times less efficiently in U251MG astrocytoma than in HeLa cells. In contrast, the HIV-1 and the SV40 promoters were equally expressed in both cell lines, indicating that U251MG cells do not have a general defect in expression but a specific transcriptional inhibition of TRBP promoter. Furthermore, the similar HIV promoter activity in U251MG versus

![Image](http://www.jbc.org/)

**Fig. 6.** TRBP1 and TRBP2 promoters are weakly active in astrocytic U251MG compared with HeLa cell lines. Luciferase activity from cellular extracts of HeLa (black boxes) or U251MG (white boxes) cells transfected with pGL3 plasmids containing no promoter (lane 1), SV40 (lane 2), HIV-1 LTR (lane 3), TRBP1 and TRBP2 (lane 4), TRBP1 (lanes 5–10), or TRBP2 (lanes 11–14) promoter. TRBP1 promoter starts from nucleotide −1397 (lane 5), −1255 (lane 6), −855 (lane 7), −587 (lane 8), −461 (lane 9), or −330 (lane 10). TRBP2 promoter starts from nucleotide −330 (lane 11), −244 (lane 12), −127 (lane 13), or +38 (lane 14). The luciferase activity was normalized to Renilla activity. The given values represent the averages of three independent experiments performed in triplicate (± S.E.).

**Fig. 7.** Semi-quantitative RT-PCR analysis on the endogenous TRBP1 and TRBP2 mRNA. 5 μg of total RNA from HeLa (lanes 1 and 4), Jurkat (lanes 2 and 5), and U251MG (lanes 3 and 6) were reverse transcribed into cDNA, and PCR analysis was performed with specific primers for TRBP1, TRBP2, and GAPDH as described under “Experimental Procedures.” The histogram represents the densitometric scanning of the gel shown after normalization to GAPDH.
Two Promoters in the Human tarbp2 Gene

48813

HeLa cells confirms that the lack of HIV replication in astrocytic cells is not due to an absence of LTR expression from U3-TAR sequences but to a specific reduced translation of proteins produced from unspliced RNA. This observation correlates with the poor expression of Gag, Pol, and Env proteins in astrocytes, whereas Tat and Rev are expressed at high levels (19, 21). Our results are compatible with a deficit in TRBP promoter expression that subsequently will produce a low PKR activity, a decreased translation efficiency, and a low viral replication. The identification of the different factors that influence TRBP promoter expression would help to elucidate its specific regulation. The modulation of TRBP expression through its promoter may be a way to control HIV expression and replication in cells.

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Organization of the Human \textit{tarbp2} Gene Reveals Two Promoters That Are Repressed in an Astrocytic Cell Line
Sylvie Bannwarth, Lily Talakoub, Franck Letourneur, Mariela Duarte, Damian F. Purcell, John Hiscott and Anne Gatignol

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