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Interaction of the Epstein-Barr Virus mRNA Export Factor EB2 with Human Spen Proteins SHARP, OTT1, and a Novel Member of the Family, OTT3, Links Spen Proteins with Splicing Regulation and mRNA Export*

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The Epstein-Barr virus early protein EB2 (also called BMLF1, Mta, or SM), a protein absolutely required for the production of infectious virions, shares properties with mRNA export factors. By using a yeast two-hybrid screen, we have identified the human protein OTT3 as an EB2-interacting factor. OTT3 is a new member of the Spen (split end) family of proteins (huSHARP, huOTT1, DmSpen, and muMINT), which are characterized by several N-terminal RNA recognition motifs and a highly conserved C-terminal SPOC (Spen Paralog and Ortholog C-terminal) domain that, in the case of SHARP, has been shown to interact with SMRT/NCoR co-repressors. OTT3 is ubiquitously expressed as a 120-kDa protein. Transfected OTT3 is a nonshuttling nuclear protein that co-localizes with co-transfected EB2. We also showed that EB2 interacts with the SPOC domains of both OTT1 and SHARP proteins. Although the OTT3 interaction domain maps within the 40 N-terminal amino acids of EB2, OTT1 and SHARP interact within the C-terminal half of the protein. Furthermore, we demonstrated that the capacity of the OTT3 and OTT1 SPOC domains to interact with SMRT and repress transcription is far weaker than that of SHARP. Thus there is no evidence for a role of OTT3 in transcriptional regulation. Most interestingly, however, we have found that OTT3 has a role in splicing regulation; OTT3 represses accumulation of the alternatively spliced β -thalassemia mRNAs, but it has no effect on the β -globin constitutively spliced mRNA. Thus our results suggested a new function for Spen proteins related to mRNA export and splicing.

In eukaryotic cells, an intensely active bidirectional transfer of RNAs and proteins occurs between the nucleus and the cytoplasm. Several

cellular factors involved in the export of mRNAs have been identified, including UAP56 (yeast Sub2p), REF (yeast Yra1p), and TAP (yeast Mex67p), that play a major role in the nuclear export of most mRNAs generated from intronless and intron-containing genes (for reviews see Refs. 1–3). Despite the profusion of cellular mRNA export factors available, some viruses infecting mammalian cells express virus-encoded factors essential for the export of a specific subset of viral mRNAs. These factors include human immunodeficiency virus Rev (4, 5), human T-cell lymphotropic virus type I, Rex (6, 7), herpes simplex virus type 1, ICP27 (8), and the Epstein-Barr virus (EBV)⁷ protein EB2 (9). EB2 is a nuclear protein that shuttles between the nucleus and the cytoplasm in a CRM1-independent manner (10–13). It binds RNA both *in vitro* (14) and *in vivo* (14, 15), and in transient expression assays, it induces the cytoplasmic accumulation of mRNAs generated from intronless genes or unspliced mRNAs generated from intron-containing genes (12). More importantly, epithelial cells carrying an EBV genome with the EB2 gene deleted do not produce infectious virions, and this appears to be due to the inefficient nuclear export of a specific subset of early and late viral mRNAs (9). However, the molecular mechanisms by which EB2 exports nuclear mRNAs to the cytoplasm remain elusive.

Evidence that DNA transcription, mRNA processing, and mRNA export are coupled in yeast and mammalian cells has accumulated recently (for review see Refs. 16 and 17). In yeast, recruitment of nuclear export factors to nascent messenger ribonucleoproteins (mRNPs) appears to occur by direct interaction with RNA polymerase II (18) or through interaction with the yeast elongation complex THO to form the TREX complex (TRanscription EXport complex) (19). The critical factors that are likely to be loaded onto nascent mRNPs during transcription are the yeast factors Sub2p and Yra1p (20–22) or their mammalian homologs, respectively, UAP56 and Aly/REF (23). However, splicing appears to largely influence the efficiency of mRNA export in mammalian cells. The first clue that splicing and mRNA export are coupled came from micro-injections studies of *Xenopus* oocytes, showing that splicing enhances the efficiency of mRNA export (24). These results were confirmed by the identification, in metazoans, of the exon junction complex, a multiprotein complex deposited some 20–24 nucleotides upstream of the exon-exon junction (25). Ultimately, exon junction

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⁷ The abbreviations used are: EBV, Epstein-Barr virus; RRM, RNA recognition motifs; aa, amino acids; ORF, open reading frame; mAb, monoclonal antibody; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; hnRNP, heterogeneous nuclear ribonucleoprotein; TRITC, tetramethylrhodamine isothiocyanate; mRNPs, messenger ribonucleoproteins; hu, human; mu, murine; Dm, *Drosophila*; NLS, nuclear localization signal; Cter, C-terminal; Nter, N-terminal; DBD, DNA binding domain.

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complex-associated mRNPs are directed to the nuclear pore complex by the interaction between Aly/REF and the soluble mRNA export heterodimer TAP/p15 (26) (Mex67p/Mtr2p in yeast) (27). Furthermore, it has been shown recently that recruitment of the mammalian TREX complex (unlike that of the yeast TREX) is only indirectly coupled to transcription through splicing (28). However, it must be emphasized that in yeast and mammalian cells, some mRNAs generated from intronless genes are also efficiently exported. Most interestingly, Sub2p (20), Yra1p (29), and Mex67p (30) in yeast and mammalian splicing factors SRp20, 9G8 (31, 32), and U2AF (33) are essential for the export of these mRNAs transcribed from intronless genes through their interaction with TAP/p15.

What emerges from the above findings is that splicing factors are essential for the loading of nuclear export factors onto mRNPs generated both from intron-containing and intronless genes. Moreover, in recent years, several transcriptional co-regulators have been found to play a role in other steps of gene expression, in particular splicing (for a review see Ref. 34). Among these transcriptional co-regulators, SHARP and the related protein OTT1 co-purify with the spliceosome (35), suggesting that they may be involved in splicing regulation and could participate in the loading of export factors onto mRNPs. SHARP (36) and OTT1 (also called RBM15) (37, 38) are members of the Spen (split end) family of nuclear proteins. These proteins are conserved in man (huSHARP and huOTT1/RBM15), mouse (MuMint), *Drosophila* (DmSpen), and *Caenorhabditis elegans* (CeSpen) (39). They are characterized by the presence of several RNA recognition motifs (RRMs) located at their N terminus and by a C-terminal conserved SPOC (Spen Paralog Ortholog C-terminal) domain. Recent crystallographic studies (39) suggest that the SPOC domain of the Spen proteins directly interacts with SMRT/NCoR co-repressors and may have an essential function in repression of transcription. Indeed, Spen proteins have a repressor function in several signaling pathways. DmSpen is a tissue/promoter-specific regulator of Wingless signaling in larval tissues (40) and regulates neuronal cell fate and axon extension in the *Drosophila* embryo by reducing the level of the repressors Suppressor of Hairless and Yan, which are essential for Notch and epidermal growth factor receptor signaling, respectively (41, 42). SHARP is a component of co-repressor complexes recruited to the steroid receptor (36). It also interacts with RBP- κ , which mediates Notch signaling (43). MINT binds to and represses the function of Msx2, a homeodomain transcriptional repressor involved in skeletal and neural development (44). Taken together, these data suggest that the Spen proteins, in the course of their co-repressor function, may potentially bind to RNA through interaction with spliceosome components.

Here we have characterized a novel human Spen protein, OTT3, by virtue of its interaction with the EBV mRNA export factor EB2. OTT3 has three N-terminal RRM domains and a C-terminal SPOC domain and is ubiquitously expressed. Most interestingly, we found that EB2 also interacted with the two other members of the Spen family known in man, SHARP and OTT1. SHARP was described previously as a transcriptional repressor by interacting with the SMRT/NCoR co-repressor via its SPOC domain. We found that despite the similarity of structure of the SPOC domains of the three human proteins, OTT3, OTT1, and SHARP, only SHARP can efficiently recruit the SMRT co-repressor and repress transcription via this domain. Finally, we have shown that OTT3 functions in the repression of alternative splicing.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The yeast two-hybrid screen was carried out using the MATCHMAKER system (Clontech) according to the

manufacturer's instructions. An EBV-transformed human peripheral lymphocyte two-hybrid cDNA library and pGBT9-EB2 were co-transfected into the yeast strain YRG2. Yeast transformants were selected on Leu-Trp-His-depleted DOBA medium (Bio 101, Inc.) for 4 days at 30 °C. Clones positive for histidine expression were further tested for β -galactosidase activity using the classical colony lift filter assay. Clones positive in this second assay were selected and subjected to sequencing.

Plasmids—A partial OTT3 cDNA containing the whole OTT3 coding sequence was reconstructed by assembling two I.M.A.G.E. Consortium/LLNL EST clones, BF309623 and AI910922. Clone BF309623 digested with EcoRI and XhoI and clone AI910922 digested with XhoI and XbaI were ligated in a three fragment reaction into a polylinker-modified version of the pSG5 vector (Amersham Biosciences) linearized with EcoRI and SpeI to give plasmid pSG5-OTT3. pSG5FLAG-OTT3 was constructed by PCR amplification of the OTT3 ORF and insertion as a fusion with the FLAG epitope into the polylinker of a modified version of pSG5FLAG. The following eukaryotic expression plasmids are all CMV immediate-early promoter-based vectors (pCI from Promega). When F precedes the name of the protein, the corresponding protein was tagged at its N terminus with a FLAG epitope, which can be detected with the monoclonal antibody M2 (Sigma). pCIF.EB2 contains the intronless *BSLF2/BMLF1* cDNA. Mutants F.EB2. Δ D1 (14) and F.NLS.EB2.Cter (13) have been described previously. In pCIF.EB2. Δ B, EB2 is deleted from aa 61 to 124. The deletion was generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) and the oligonucleotide primer 5'-GAGATTTTCATCCTCAGAGGAGGAGGGCCACAAAAGGCGACGCGG-3' and its complement on the opposite strand. In pCIF.EB2. Δ D, EB2 is deleted from aa 181 to 260. The deletion was generated as described above but with the oligonucleotide primer 5'-GCAACAGAGCAACCAGAGGTAAAGTGCAACCCAGACACGAG-3' and its complement on the opposite strand. pCIF.EB2.N-ter contains the 184 N-terminal aa of EB2 and was generated by replacing an XhoI-XbaI fragment from pCIF.EB2 with a PCR fragment amplified from pCIF.EB2 using the following oligonucleotides: 5'-TGCCTCGAGTTCCTTCTCAGAGAC-3' and 5'-TGCTCTAGACTATTCTGACCGGGGACC-3' and subsequently cut by XhoI-XbaI. pCIF.EB2.M1 has the 50 aa at the N-terminal end deleted. pSG5F.SMRT.VP16 was generated by subcloning a PCR-amplified DNA fragment containing sequences coding for the region between aa 981 and the C-terminal of human SMRT (45) into pSG5F.NLS.VP16, making a fusion with the SV40 T antigen NLS and the VP16 activation domain. Plasmid Gal4-Sharp SID (36), which contains the SPOC domain of Sharp in fusion with the Gal4 DNA-binding domain, was a gift from the laboratory of Dr. R. M. Evans. The regions coding for the SPOC domains of OTT3 and OTT1 were specifically PCR-amplified using the following oligonucleotides: 5'-GCCCTCGAGCCGAGCCCAAGCCTCTGG-3' and 5'-GCCGGATCCTAGGCA-GTGTCTCTGACG-3' for OTT3 and 5'-GGCCTCGAGCCAAGCTGAAGTCCCCGTCCAG-3' and 5'-GCCGGATCCTTAGGACGCCACCGACAATG-3' for OTT1 and then subcloned into the XhoI-BamHI sites of pG4MpolyII (46). The reporter plasmid pG4-TK-CAT has been described elsewhere (47). pGEX-OTT3 was generated by subcloning a BamHI-NotI (partial digest) fragment containing the whole OTT3 ORF between the BamHI and NotI site of pGEX4T-1 (Amersham Biosciences). For expression in bacteria of the SPOC domains of OTT3, OTT1, or Sharp in fusion with GST, PCR-amplified DNA fragments containing the coding sequences for aa 722–890 (OTT3), aa 643–957 (OTT1), or aa 3498–3664 (Sharp) were subcloned in a pGEX type vector (Amersham Biosciences). pEGFPC1 REF2-II (48) was a gift

from the laboratory of Dr. E. Izaurralde. Plasmids pUC β 128SV and pUC β Δ 128SV were kindly provided by A. Krainer (49).

Transfections and Heterokaryon Assays—HeLa cells and NIH3T3 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. HeLa cells were seeded at 1×10^6 cells per 100-mm diameter Petri dish 10 h prior to transfection. Transfections were performed by the calcium precipitate method. Plasmids used for transfections were prepared by the alkaline lysis method and purified through two CsCl gradients. For the heterokaryon assays, 24 h post-transfection the precipitate was washed, and cells were trypsinized, and $\sim 2 \times 10^5$ HeLa cells were seeded on glass coverslips with an equal number of NIH3T3 cells in 35-mm dishes. The cells were allowed to grow overnight and were then treated for 2 h with 100 μ g/ml of cycloheximide to inhibit protein synthesis. Subsequently, cells were washed with phosphate-buffered saline (PBS), and the heterokaryon formation was carried out by incubating the coverslips for 2 min in 50% PEG 3000–3700 (Sigma) in PBS. Following cell fusion, coverslips were washed extensively in PBS and returned to fresh medium containing 100 μ g/ml of cycloheximide. After 2 h at 37 °C, cells were fixed with 4% paraformaldehyde, and indirect immunofluorescence was performed, essentially as described previously (12).

Northern Blotting—The Northern blot was hybridized with a 672-bp long 32 P-labeled probe, covering the end of the OTT3 reading frame.

Western Blotting and Immunofluorescence Assays—Western blotting was performed as described previously (9). Membranes were incubated with either the anti-FLAG M2 monoclonal antibody (mAb) (Sigma), an anti-OTT3 mAb (1D2) that we have generated following mouse immunization with a GST-OTT3 fusion protein, or an anti-Gal4 DNA binding domain (DBD) mAb (RK5C1) (Santa Cruz Biotechnology). For indirect immunofluorescence experiments, we used either the anti-FLAG M2 mAb or an anti-hnRNP-C mAb (4F4, kindly provided by Dr. G. Dreyfuss) (50) or an anti-EB2 polyclonal rabbit antibody (51). An Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (H + L; Interchim) or a TRITC-conjugated anti-mouse antibody (Jackson ImmunoResearch) was used as a secondary antibody, and the nuclei of the cells were stained by incubation with Hoechst 33258 (Sigma) solution at 5 μ g/ml.

Immunoprecipitations—For the co-immunoprecipitations of transiently expressed OTT3 and EB2 proteins, HeLa transfected cells were harvested from 100-mm dishes 48 h post-transfection and lysed in 1 ml of IP buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.5% Nonidet P-40) plus protease inhibitors (Roche Applied Science). Cell extracts were incubated with a rabbit anti-EB2 polyclonal antibody (51) for 4 h at 4 °C. For immunoprecipitation of the endogenous OTT3 protein, epithelial and B-cells were lysed in 1 ml of RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS). Cell extracts were incubated with the anti-OTT3 mAb (1D2) for 2 h at 4 °C and then with a rabbit anti-mouse IgG (Amersham Biosciences) for 1 h at 4 °C. The protein-antibody complexes were purified using 30 ml of protein A-Sepharose beads (Amersham Biosciences). Immunopurified proteins were analyzed by Western blotting.

In Vitro GST Pull-down Assays—GST and GST fusion proteins were expressed in *Escherichia coli* BL21 codon plus strain and affinity-purified by glutathione-Sepharose 4B beads (Amersham Biosciences). 35 S-Labeled F.EB2, F.EB2 mutants, and SMRT-VP16 were produced in a rabbit reticulocyte lysate *in vitro* transcription/translation TNT system (Promega). Binding reactions were carried out in 500 ml of MTPBS buffer (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 mM EDTA, 1% Triton X-100, pH 7.3) for 1 h at 4 °C, and then complexes bound to the glutathione-Sepharose 4B beads were washed five times in

the same buffer. Proteins were eluted in SDS-PAGE loading buffer and analyzed by SDS-PAGE. The bands revealed by autoradiography were subsequently quantified using a Storm PhosphorImager.

Quantification of CAT Protein—CAT protein expression was evaluated by using the CAT enzyme-linked immunosorbent assay (Roche Applied Science).

RNA Purification and RT-PCR—Cytoplasmic RNAs were prepared by resuspending cells in 475 μ l of Lysis buffer (10 mM Tris-HCl, pH 7.8, 10 mM NaCl, 2 mM MgCl₂, 5 mM dithiothreitol) that was kept in ice for 5 min before the addition of 25 μ l of 10% Nonidet P-40. After 5 min of incubation in ice, the nuclei were recovered by centrifugation. RNA was extracted from both the nuclei and the cytoplasmic fraction by using the Qiagen RNeasy kit following the manufacturer's instructions. 2 μ g of RNA was reverse-transcribed using oligo(dT)_{12–18} and the Superscript II reverse transcriptase (Invitrogen). β -Globin and β -thalassemia transcripts were PCR-amplified from the reverse-transcribed cDNA using the following primers: pA (5'-CATTGCTTCTGACACAAGT-3') and pB (5'-GTGCAGCTCACTCAGTGTGGC-3'). Actin transcripts were PCR-amplified using the following primers: 5'-GCTGCGTGTG-GCTCCCGAGGAG-3' and 5'-ATCTTCATGTGCTGGGTGCCA-G-3'. Each PCR was performed in the presence of 1 μ Ci of [α - 32 P]dCTP, and the amplified fragments separated on 5% polyacrylamide gel were visualized by autoradiography. The conditions of the PCR were chosen so as to be in the linear range of the amplification evaluated by using various dilutions of the reverse-transcribed cDNA.

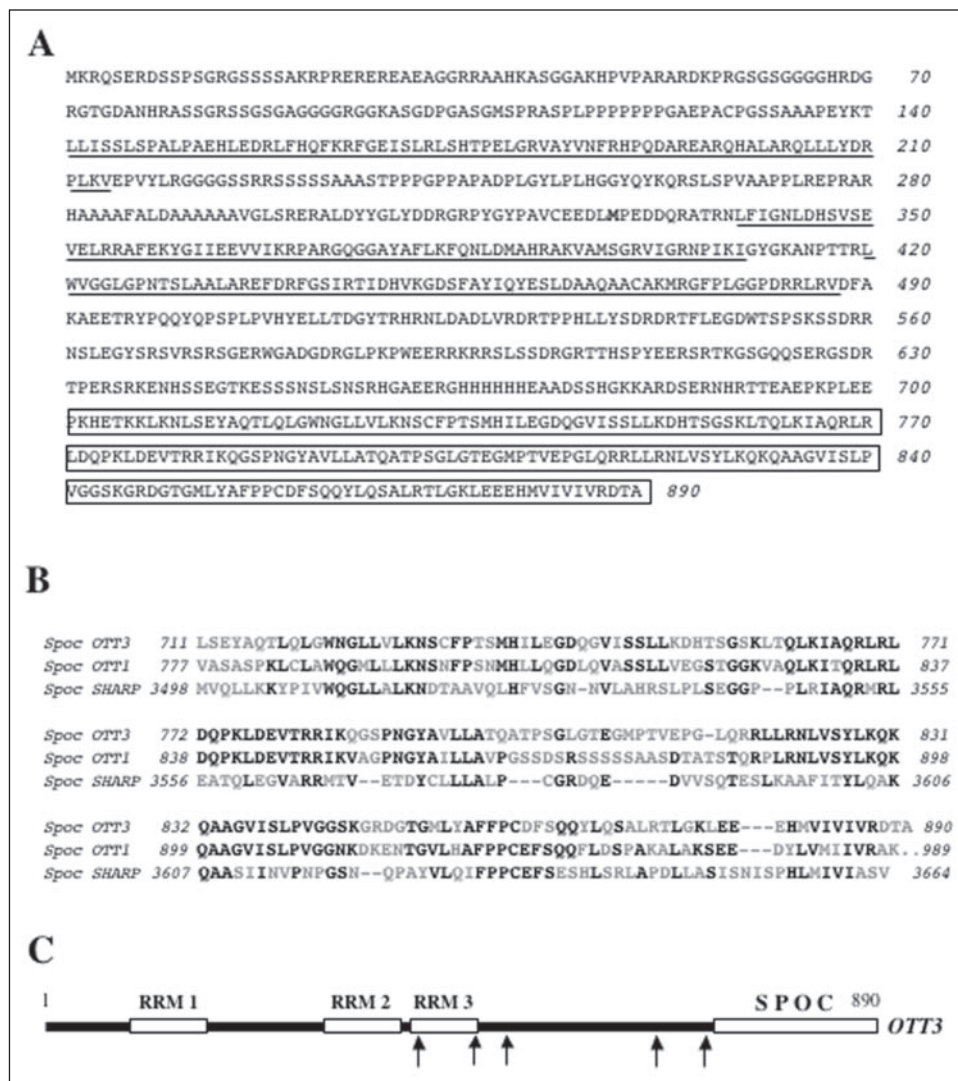
RESULTS

HuOTT3 Is an EB2-interacting Protein—In order to isolate cofactors for the EBV EB2 protein, a yeast two-hybrid screen was performed using EB2 as a bait, and a human B-cell line cDNA library, which was cloned in pACT. We selected eight cDNA clones that all contained sequences identical to the 3'-end of the human chromosome 3p21.1 gene deposited in the GenBank™ data base under the name HUMAGCGB (accession number NM013286). Although a 563-aa-long protein sequence was proposed to be the HUMAGCGB gene product because the DNA sequence located upstream of the proposed start codon was relatively extensive and not interrupted by stop codons in the same frame, we suspected that the ORF could be longer. Therefore, we searched for additional 5'-sequences in the htgs and dbest data bases. Analysis of sequences found in the htgs data base and that overlapped with the 3p21.1 sequence allowed us to find a putative initiation codon 981 bp upstream from the first methionine of the protein described previously, with stop codons and promoter-like sequences lying upstream of this new ATG. A Blast search with the putative 890-amino acid protein sequence (Fig. 1A) revealed that it was related to the Spen (Split ends) family of proteins and had previously been named OTT3 following its identification in the data bases as being closely related to the OTT1 protein (37). Spen proteins have been identified in *C. elegans*, *Drosophila*, and mammals (39). They are characterized by three N-terminal RRM domains and a conserved SPOC domain. OTT3 contains three N-terminal RRM domains (Fig. 1, A and C) and a conserved C-terminal SPOC domain (Fig. 1, B and C). The alignments of the SPOC domain of OTT3 with those of OTT1 and SHARP using ClustalW (52) (Fig. 1B) shows 58% of identity with OTT1 and 30% of identity with SHARP, suggesting that OTT1 and OTT3 are more closely related.

The interaction between OTT3 and EB2 was further examined in a co-immunoprecipitation assay in mammalian cells. The OTT3 cDNA that we reconstituted as described under "Experimental Procedures" was tagged at its N terminus with the FLAG epitope (F.OTT3) and was expressed in HeLa cells either alone or together with FLAG-tagged EB2

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FIGURE 1. Amino acid sequence of a novel protein of the Spen family. A, HuOTT3 amino acid sequence. The previously proposed start codon is indicated in **boldface**. The three domains of homology with RRM s are underlined. The SPOC homology domain is **boxed**. B, amino acid alignment of the SPOC domains of huOTT3 with huSHARP and huOTT1. Identical residues are indicated in **black letters**. C, schematic representation of huOTT3; the three RRM s and the SPOC domain are indicated by **white boxes**. **Arrows** indicate the location of the N terminus of the partial OTT3 peptides encoded by the different cDNA clones obtained in the two-hybrid screen.



(F.EB2) or EB2 with its RNA-binding domain and REF-interacting region both deleted (F.EB2.ΔD) (14) (Fig. 2A). The EB2 proteins were immunoprecipitated using a rabbit polyclonal antibody directed against the N terminus of EB2. Co-immunoprecipitated F.OTT3 proteins were detected by Western blotting using an anti-FLAG mAb (M2, Sigma). F.OTT3 is detected at a molecular mass of 120 kDa in the lysates of cells transfected with an expression plasmid for F.OTT3 either alone or in combination with expression plasmids for F.EB2 or F.EB2.ΔD (Fig. 2B, lanes 7–9). Because they are tagged with the FLAG epitope, F.EB2 and F.EB2.ΔD are also detected by the anti-FLAG mAb in the lysates where they have been expressed (Fig. 2B, lanes 8 and 9, respectively). As shown in Fig. 2B, no protein was immunoprecipitated in the absence of anti-EB2 polyclonal antibody (lanes 1, 3, and 5). F.OTT3 was not directly immunoprecipitated by the anti-EB2 antibody (Fig. 2B, lane 2) but was efficiently co-immunoprecipitated from lysates of HeLa cells co-transfected with the F.EB2 expression construct (lane 4), suggesting an interaction of huOTT3 and EB2 *in vivo*. A similar interaction was also observed between OTT3 and F.EB2.ΔD, which has the EB2 RNA-interacting domain deleted (Fig. 2B, lane 6). This suggests that the interaction between OTT3 and EB2 is not mediated by the RNA.

The 51 Amino Acids at the EB2 N-terminal Interact with the huOTT3 SPOC Domain—It is noteworthy that among the polypeptides corresponding to the cDNAs identified by the two-hybrid screen, most did

not contain an RRM (the extremity of each of these cDNAs is indicated in Fig. 1C). However, they all contain a complete SPOC domain suggesting that this region could mediate the interaction with EB2. In order to confirm the specificity of interaction between EB2 and the SPOC domain of huOTT3, we performed *in vitro* binding assays using GST-SPOC.OTT3 and [³⁵S]methionine-labeled EB2 or EB2 deletion mutants (Fig. 3A). The [³⁵S]methionine-labeled EB2 proteins were produced by *in vitro* transcription/translation. As shown in Fig. 3B, EB2 bound to GST-SPOC.OTT3 (lane 2) but not to GST (lane 3). In order to rule out the possibility that EB2 binding to RNA is required for interaction with SPOC.OTT3, we used the EB2.ΔD1 protein with the RNA-binding domain deleted (RNA-binding domain (*RBD*), Fig. 3A) (14). As shown in Fig. 3B, EB2.ΔD1 bound to GST-SPOC.OTT3 (lane 6) but not to GST (lane 5). Thus, the interaction between huOTT3 and EB2 is likely to be direct.

Among the other EB2 mutants examined, EB2.Nter (Fig. 3B, lanes 10–12) interacted with GST-SPOC.OTT3, whereas EB2.Cter (lanes 7–9) did not, suggesting that the interaction domain is located in the 184 aa at the N terminus of EB2. Analysis of two additional deletion mutants, EB2.M1 (Fig. 3B, lanes 13 and 14) and EB2.ΔB (lanes 16–18), further suggested that the interaction domain could be restricted to the first 51 aa at the N terminus.

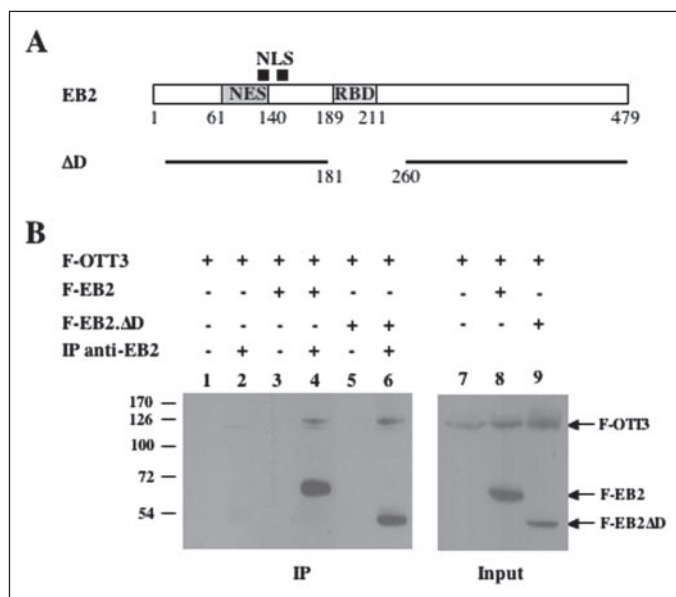


FIGURE 2. HuOTT3 and EB2 interact *in vivo*. *A*, schematic representation of the EB2 protein. Regions responsible for the shuttling of the protein between the nucleus and the cytoplasm (nuclear export signal (NES) and nuclear localization signal (NLS)) and for the binding of the protein to RNA (RNA-binding domain (RBD)) are indicated. The ΔD mutant is represented below. *B*, *in vivo* co-immunoprecipitation of OTT3 and EB2. FLAG-tagged OTT3 was expressed either alone or together with FLAG-tagged EB2 or FLAG-tagged EB2 ΔD into HeLa cells. The cell lysates were immunoprecipitated (IP) with an anti-EB2 rabbit polyclonal antibody (lanes 2, 4, and 6) or mock immunoprecipitated (lanes 1, 3, and 5). The immunoprecipitates were immunoblotted with an anti-FLAG monoclonal antibody. Cell lysates input were included in the anti-FLAG blot (lanes 7–9).

Expression of OTT3 mRNA and Protein—The expression of OTT3 mRNA was detected by Northern blot analysis in all human tissues tested as a relatively abundant 3.5-kb mRNA transcript and a minor species of 7.5 kb (Fig. 4A). Two mRNA sequences recently deposited in GenBankTM, both under the name RBM15B mRNA, are likely to correspond to the 7.5- and 3.5-kb mRNA species, respectively (53). The first mRNA sequence (GenBankTM accession number NM013286) corresponds to a 6620-base-long reconstituted mRNA. It contains the complete OTT3 ORF and a large untranslated 3'-region. The second sequence (GenBankTM accession number BC001367) corresponds to a complete cDNA sequence (cDNA clone IMAGE:3051463) of 1693 bases. This cDNA sequence is partially co-linear to the 6620-nucleotide mRNA but starts in the middle of the OTT3 ORF and contains a poly(A) track 324 bp downstream of the end of the OTT3 ORF. Although partial, this cDNA sequence is likely to correspond to the 3.5-kb species. In order to detect the OTT3 protein in human cells, we generated a monoclonal antibody (mAb 1D2). This mAb specifically detects a protein of 120 kDa in HeLa cells transfected with pSG5.F.OTT3, an expression plasmid for the OTT3 ORF tagged with the FLAG epitope (Fig. 4B, lane 4). As expected, a protein of the same size was detected with the anti-FLAG M2 mAb (Fig. 4B, lane 2). However, because we did not detect the endogenous protein in the nontransfected HeLa cells with the mAb 1D2 (Fig. 4B, lane 3), we decided to submit the cell lysates to immunoprecipitation with the mAb 1D2 prior to protein analysis by Western blotting. The expression of the endogenous protein was tested in two human epithelial cells, HeLa and 293T, and EBV-positive Burkitt's lymphoma cells, Raji and Akata. A protein with an apparent molecular mass of around 120 kDa was immunoprecipitated and recognized by the mAb 1D2 in all cell line protein extracts tested (Fig. 4C). This protein is similar in size to the protein expressed from the transfected plasmid pSG5.F.OTT3 (Fig. 4C, lane 1). However, OTT3 immunoprecipitated from 293T cell extracts (Fig. 4C, lane 5) migrates slightly slower than

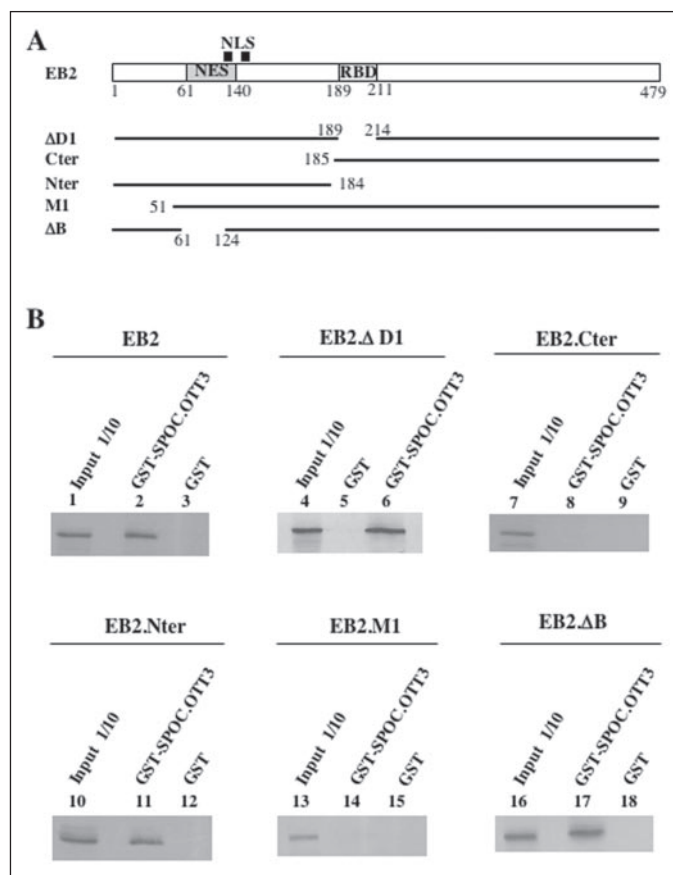


FIGURE 3. EB2 binds directly OTT3 *in vitro*. *A*, schematic representation of EB2 and the various EB2 deletion mutants. *B*, ³⁵S-labeled full-length EB2 or the various deletion mutants depicted in *A* were incubated with purified GST or GST-SPOC.OTT3 fusion bound on glutathione-agarose beads. The bound proteins were analyzed by SDS-PAGE and visualized by autoradiography.

OTT3 immunoprecipitated from HeLa, Raji, or Akata cell extracts (Fig. 4C, lanes 3, 7, and 9). This 120-kDa protein is likely to be OTT3 as our monoclonal antibody did not recognize either OTT1 or SHARP, the two proteins of the Spen family previously described in man, when transiently expressed from a plasmid (data not shown).

Taken together, these results demonstrate the following. (i) The mRNA for OTT3 is ubiquitously expressed in all human tissues tested. (ii) The product of the gene is a 120-kDa protein that corresponds in size to the protein produced from our reconstituted OTT3 ORF.

OTT3 Is a Nonshuttling Nuclear Protein That Co-localizes with EB2 in the Nucleus—The endogenous OTT3 protein was difficult to detect by indirect immunofluorescence (data not shown). The subcellular localization of OTT3 was therefore analyzed after transient expression in HeLa cells. The OTT3 protein was localized in the nucleoplasm of HeLa cells with a granular staining pattern and was excluded from the nucleoli (Fig. 5a). This is reminiscent of the nuclear localization described previously for SHARP (36). When OTT3 and EB2 were co-expressed, a similar distribution of OTT3 was observed (Fig. 5, e and j), and the confocal analysis revealed a co-localization of the two proteins in well defined foci (Fig. 5, g and k).

Because OTT3 and EB2 physically interact and EB2 is a shuttling protein, we assessed whether OTT3 is also a shuttling protein by performing a human-mouse heterokaryon assay. In this assay, OTT3 was first expressed in HeLa cells and then the cells were co-cultivated overnight with mouse NIH3T3 cells. The cells were then fused by incubation with polyethylene glycol in the presence of cycloheximide to suppress *de*

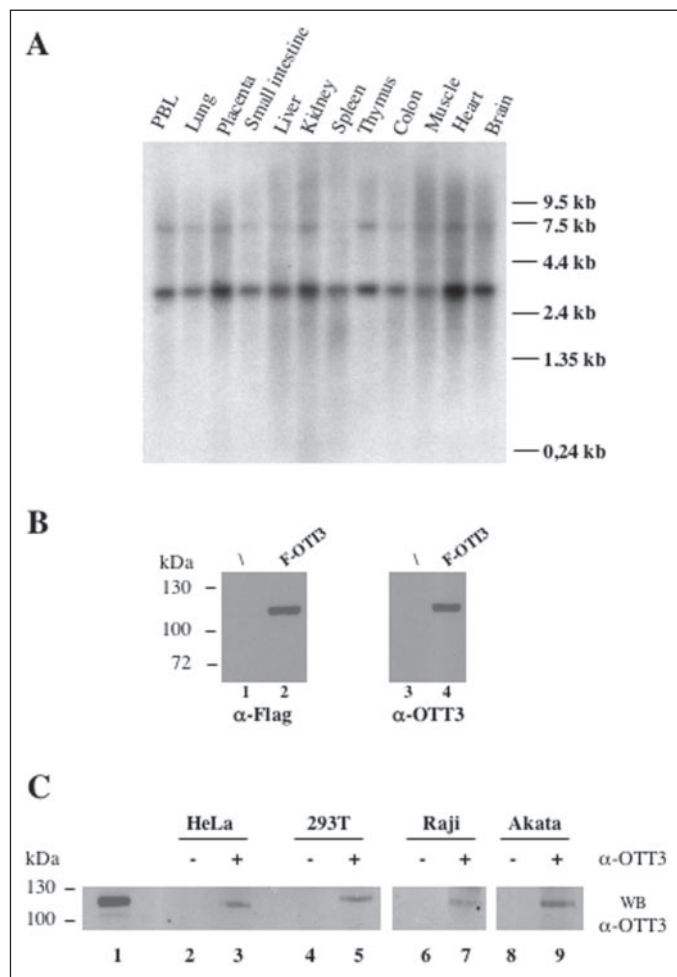


FIGURE 4. Expression pattern of huOTT3. *A*, Northern blot analysis of OTT3 expression in human tissues by using a probe located in the coding sequence. *PBL*, peripheral blood lymphocytes. *B*, FLAG-OTT3 was transiently expressed in HeLa cells from a transfected expression plasmid. The cell lysates were immunoblotted using either the anti-FLAG monoclonal antibody (lanes 1 and 2) or the specific anti-OTT3 monoclonal antibody 1D2 (lanes 3 and 4). *C*, endogenous huOTT3 is detected in both epithelial and lymphoid cell lines. Cell lysates of the epithelial HeLa and 293T cells or of the lymphoid Raji and Akata cells were first immunoprecipitated with a specific anti-OTT3 monoclonal antibody then analyzed by Western blotting using the same anti-OTT3 monoclonal antibody. Protein extract from HeLa cells transfected with an expression plasmid for FLAG-OTT3 was included in the Western blot as a positive control (lane 1).

*nov*o protein synthesis and then incubated a further 2 h. Indirect immunofluorescence was then performed to detect the presence of the protein in the human and mouse nuclei of the HeLa-NIH3T3 heterokaryons. As shown in Fig. 6A, F.OTT3 did not shuttle between the nucleus and the cytoplasm because its presence was restricted solely to the HeLa nucleus of all observed heterokaryons (panels *a* and *b*), whereas GFP-REF, which is a shuttling protein, was found in both HeLa and NIH3T3 nuclei of the heterokaryons (panels *c* and *d*). The HeLa cell endogenous nonshuttling hnRNP-C protein was used as a negative control, and as expected was not transported from the human to the mouse nucleus (Fig. 6A, panels *e* and *f*).

We then asked whether OTT3 would shuttle in the presence of EB2. We thus co-transfected HeLa cells with expression vectors for both proteins and performed heterokaryon assays. In all the heterokaryons analyzed in which both F-EB2 and F-OTT3 were expressed, we observed that although F-EB2 was systematically found in both HeLa and NIH3T3 nuclei, F-OTT3 was restricted to the HeLa nucleus (Fig. 6B), demonstrating that even in the presence of EB2, OTT3 is not able to shuttle between the nucleus and the cytoplasm.

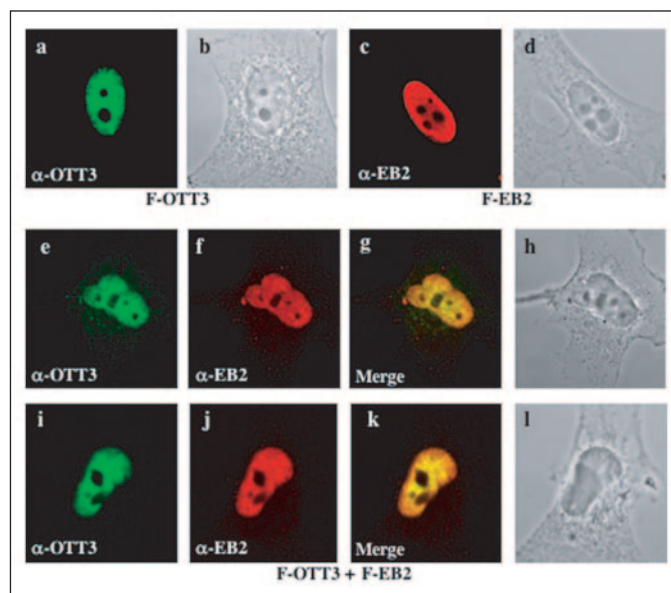


FIGURE 5. EB2 and OTT3 co-localize in the nucleus of the cells. F.EB2 and F.OTT3 were expressed in HeLa cells either alone (*a* and *b* for F.OTT3; *c* and *d* for F-EB2) or together (*e*–*l*). Cells were then immunostained using either an anti-OTT3-specific monoclonal antibody and an Alexa Fluor 488-conjugated secondary antibody (*a*) or an anti-EB2 rabbit antiserum and a TRITC-conjugated secondary antibody (*c*) or both successively (*e*–*g* and *i*–*k*). The cell fluorescence was examined using confocal microscopy. *b*, *d*, *h*, and *l* show the same cells in phase contrast microscopy.

EB2 also Interacts with the SPOC Domains of OTT1 and Sharp—Because EB2 interacts with the C-terminal SPOC domain of OTT3 and because this domain is highly conserved among the members of the Spen family, we asked whether EB2 could also interact with the SPOC domains of the other two members of the family characterized so far in man, OTT1 and SHARP. For this, we performed GST pull-down experiments using GST-SPOC-OTT1, -OTT3, or -SHARP fusion proteins. As shown in Fig. 7B, EB2 interacted efficiently with each of the GST-SPOC fusion proteins (lanes 2–4) but not with GST alone (lane 5). In order to identify the interaction domain in EB2, we performed GST pull-down assays with deletion mutants of EB2 (Fig. 7A). As shown in Fig. 7B, we confirmed that EB2.Nter but not EB2.M1 or EB2.Cter interacted with GST-SPOC-OTT3. Most surprisingly, we found that EB2.M1 and EB2.Cter interacted with both GST-SPOC-OTT1 and GST-SPOC-SHARP, but EB2.Nter did not (Fig. 7B). Thus it appears that although EB2 interacts with the SPOC domain of the three proteins, OTT1, OTT3, and Sharp, the domains of EB2 involved in these interactions are different, with the 51 N-terminal aa of EB2 involved in the interaction with SPOC OTT3 and the EB2 C-terminal domain (from aa 185) involved in the interaction with SPOC-OTT1 and SPOC-SHARP.

Differential Recruitment of SMRT by OTT1, OTT3, and SHARP SPOC Domains—The SHARP protein was characterized previously as a component of transcriptional repression complexes involved in both nuclear receptor and Notch/RBP- κ pathways, and its SPOC domain was found to interact with the universal transcriptional co-repressors SMRT and NCoR (36). The crystallographic structure of the SHARP SPOC domain (also called SID/RD domain for SMRT interaction domain/repression domain) was recently elucidated, and the residues important for interaction with SMRT were localized in a basic cluster mapped on the surface of the protein and conserved among the other Spen proteins (39). It was thus likely that both OTT1 and OTT3 SPOC domains could also recruit SMRT. To determine this, we first performed GST pull-down assays using GST-SPOC -OTT3, -OTT1, and -SHARP fusion proteins. Because huSMRT, with an estimated mass of

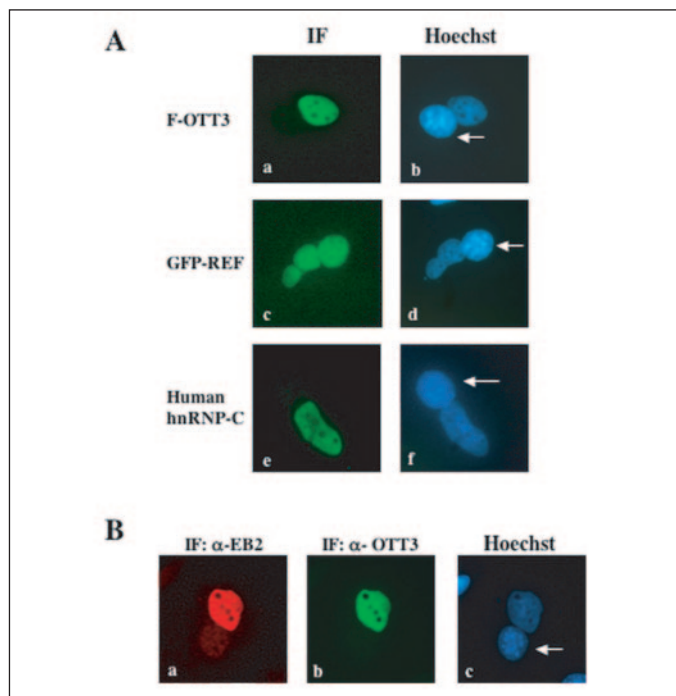


FIGURE 6. F.OTT3 is a nonshuttling protein. *A*, F-OTT3 or GFP-REF were expressed in HeLa cells. After 48 h, HeLa cells were fused with NIH3T3 cells to form heterokaryons and incubated for 2 h with medium containing cycloheximide. The cells were immunostained using either an anti-OTT3 rabbit polyclonal antibody (*panel a*) or anti-hnRNP-C (4F4) (*panel e*) monoclonal antibodies and an Alexa Fluor-conjugated secondary antibody. GFP-REF (*panel c*) is visualized directly under UV light. All the cells were stained with Hoechst dye to differentiate between the HeLa and NIH3T3 nuclei in the heterokaryons (*b*, *d*, and *f*). Arrows identify the NIH3T3 cell nuclei. *B*, F-OTT3 and F-EB2 were co-expressed in HeLa cells. Heterokaryons were formed as in *A*, and the cells were immunostained with both the rabbit polyclonal antibody against EB2 and the monoclonal antibody against OTT3 (1D2) and then with both a TRITC-conjugated secondary antibody (anti-rabbit IgG) and an Alexa Fluor-conjugated secondary antibody (anti-mouse IgG). All the cells were stained with Hoechst dye (*panel c*). EB2 (*panel a*) and OTT3 (*panel b*) are visualized in the same cells. *IF*, immunofluorescence.

168 kDa, was not produced very efficiently in an *in vitro* transcription/translation system, we used a truncated version of SMRT for these experiments. It has been shown previously that SMRT interacts with SHARP via a motif conserved among mammalian SMRT and NCoR, called the LSD (lysine-serine-aspartic acid) motif, which is located at the C terminus of these proteins. We thus subcloned the 514 C-terminal amino acids of huSMRT (45) into the eukaryotic expression vector pSG5.FNLS.VP16. The protein expressed from this construct (pSG5-FNLS.VP16-SMRT) is a fusion between the SV40 T antigen NLS, the VP16 activation domain, and the C terminus of SMRT. This construction also had the advantage that it could be subsequently used in a two-hybrid assay in mammalian cells (see below). This fusion protein, ³⁵S-labeled, was expressed *in vitro* in a rabbit reticulocyte lysate system. As shown in Fig. 8A, we found that the FNLS.VP16.SMRT interacted with the three GST-SPOC-OTT1, -OTT3, and -SHARP proteins. However, the interaction was much more efficient in the case of GST-SPOC-SHARP. Indeed, a PhosphorImager quantification of the bands showed that 30% of the input FNLS.VP16.SMRT protein was retained on the GST-SPOC-SHARP-glutathione beads, whereas only 4.5 and 3.5%, respectively, were retained on the GST-SPOC-OTT1 or -OTT3 glutathione beads. As a comparison, using the same GST-SPOC-OTT1/-OTT3/-SHARP protein preparations, we found that EB2 interacted with a similar efficiency with the three proteins (Fig. 7B), which suggested that the binding difference observed is specific for FNLS.VP16.SMRT.

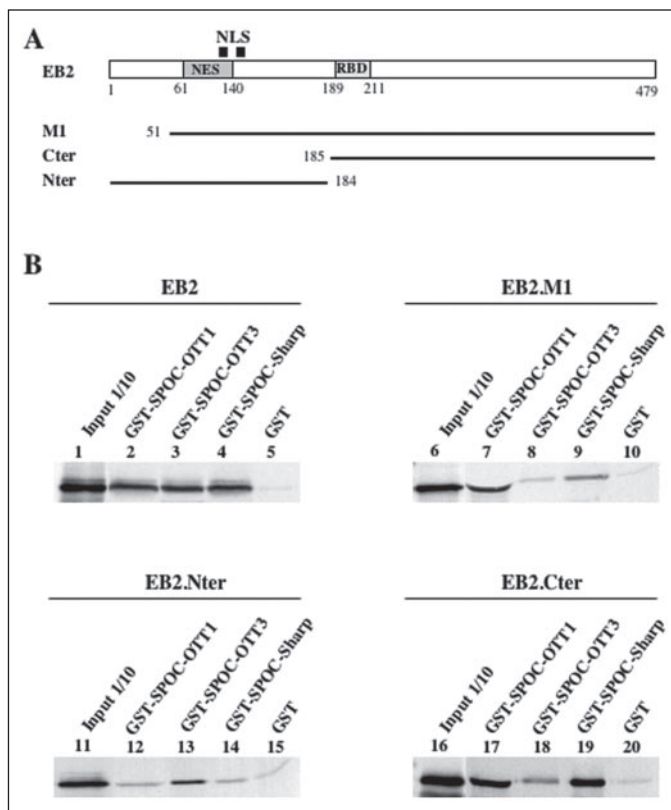


FIGURE 7. EB2 interacts with the SPOC domain of each of the three Spen family proteins OTT3, OTT1, and SHARP. *A*, schematic representation of EB2 and the various deletion mutants used in the GST pull-down experiments. *B*, ³⁵S-labeled *in vitro* translated full-length EB2, or the various deletion mutants depicted in *A*, were incubated with purified GST, GST-SPOC.OTT1, GST-SPOC.OTT3, or GST-SPOC.Sharp fusions bound on glutathione-agarose beads. The bound proteins were analyzed by SDS-PAGE and visualized by autoradiography. A tenth of each protein extract used for the interactions was analyzed on the same gel as an input quantification. *NES*, nuclear export signal; *RBD*, RNA-binding domain.

In order to assess the capacity of the OTT1, OTT3, or SHARP SPOC domains, to interact with SMRT and repress transcription *in vivo*, we fused the SPOC domains of OTT1 and OTT3 to the Gal4 DBD (Gal4-SPOC-OTT1 and Gal4-SPOC-OTT3, respectively). SPOC-SHARP fused to Gal4 DBD (Gal4-SHARP SID) was a gift from the laboratory of R. M. Evans. We analyzed the effect of the expression of these in Fig. 8C; Gal4-SHARP SID strongly repressed CAT expression from the reporter construct (CAT protein was undetectable even when the amount of protein extract used was increased) (Fig. 8C, compare *lane 1* with *lane 7*). This result confirms the capacity of the SHARP SPOC domain to repress transcription when targeted to a specific promoter via a heterologous DNA-binding domain, as described previously by Shi *et al.* (36). When we used the Gal4-SPOC-OTT1 or -OTT3 in the same assay, we only observed a weak repression effect (up to four times) on CAT expression. Expression levels of each Gal4-SPOC protein were checked by Western blotting, using an anti-Gal4 (DBD) mAb and were not significantly different (data not shown). This result suggests that on the contrary to the SPOC domain of Sharp, the SPOC domains of OTT1 or OTT3 do not efficiently recruit proteins with co-repressor activity *in vivo*. In order to show that this deficiency in the repression activity was due to a lower capacity to bind SMRT *in vivo*, we performed a two-hybrid assay in mammalian cells. For this, we co-expressed each of the three Gal4-SPOC proteins together with FNLS.VP16.SMRT_{Cter} and the reporter plasmid pG4-TK-CAT. As shown in Fig. 8C, FNLS.VP16.SMRT_{Cter} strongly activated the expression of CAT in the

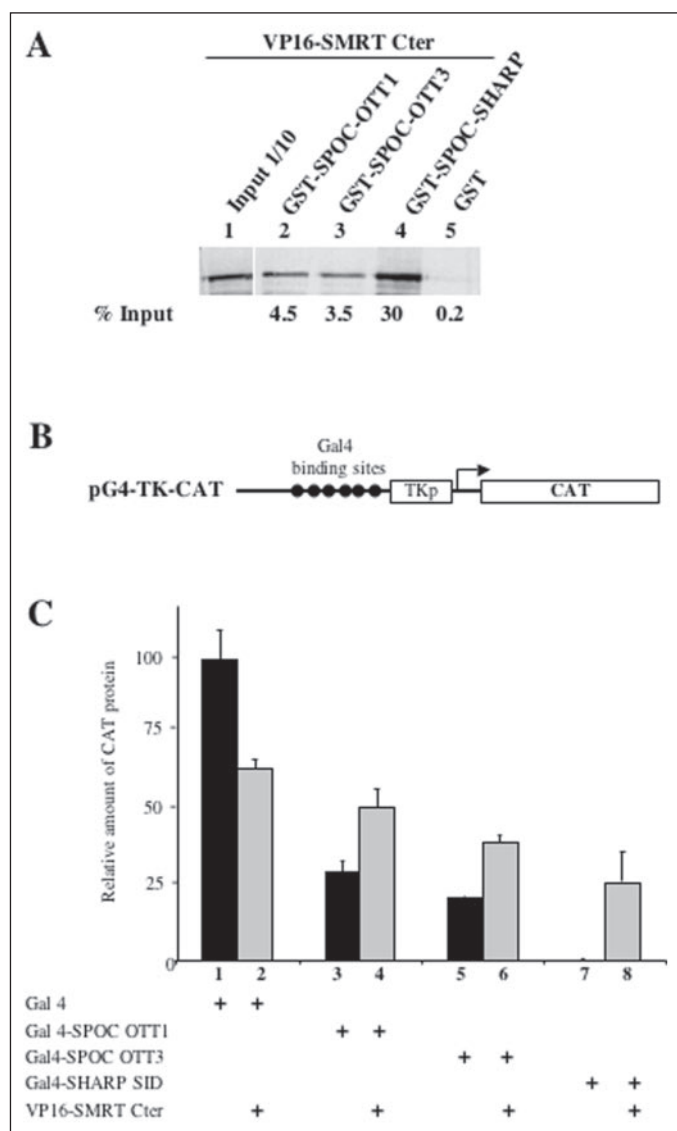


FIGURE 8. The SPOC domains of both OTT1 or OTT3 interact only inefficiently with SMRT and do not repress transcription contrary to the SPOC domain of Sharp. *A*, ^{35}S -labeled *in vitro* translated VP16-SMRT C-terminal fusion protein was incubated with purified GST, GST-SPOC.OTT1, GST-SPOC.OTT3, or GST-SPOC.Sharp fusions on glutathione beads. The bound proteins were analyzed by SDS-PAGE and visualized by autoradiography. *B*, schematic representation of the reporter construct used in the repression assay. The CAT gene is under the control of the herpes simplex virus-thymidine kinase promoter (TKp). Six Gal4-binding sites are present upstream of the TKp. *C*, HeLa cells were transfected with the pG4-TK-CAT reporter construct together with expression plasmids for Gal4, Gal4-SPOC OTT1, Gal4-SPOC OTT3, Gal4-Sharp SID, and FNLS-VP16-SMRT Cter as indicated. The amount of CAT protein expressed was quantified by CAT enzyme-linked immunoabsorbent assay. The results are expressed as relative amount of CAT protein with a fixed value of 100 given to the amount of CAT protein expressed in the presence of Gal4.

presence of Gal4-SHARP SID (compare *lane 8* with *7*), whereas it had only a weak effect on CAT expression in the presence of Gal4-SPOC OTT1 or Gal4-SPOC OTT3 (compare *lanes 4* with *3* and *6* with *5*, respectively). Together with our *in vitro* interaction data, these results suggest that only the SPOC domain of SHARP has the property to recruit SMRT efficiently and therefore strongly repress transcription.

OTT3 Specifically Represses the Accumulation of Alternatively Spliced Transcripts Versus Constitutively Spliced Transcripts—We have shown previously that EB2 induces the accumulation of unspliced mRNAs generated from genes containing cryptic 5'-splice sites such as those found in the naturally occurring β -thalassemia gene, whereas EB2

does not have any effect on constitutively spliced β -globin mRNA (54). Because OTT3 interacts with EB2, we wanted to test the effect of overexpression of OTT3 in this system. The two reporter plasmids used were the plasmid pUC β 128SV, which contains the wild-type human β -globin gene cloned under the control of the SV40 early promoter, and the plasmid pUC $\beta\Delta$ 128SV, which contains a β -thalassemia allele that has a G-to-A transition at position 1 of intron 1 which causes the activation of three cryptic 5'-splice sites otherwise completely silent in the wild-type gene (49) (Fig. 9A). These two plasmids were transiently transfected into HeLa cells either alone or together with expression plasmids for FLAG-tagged OTT3, FLAG-tagged EB2, or both. Western blot analysis using an anti-FLAG antibody demonstrated that both F-OTT3 and F-EB2 were efficiently expressed in HeLa cells (Fig. 9C). The amount of transcripts generated from either pUC β 128SV or pUC $\beta\Delta$ 128SV were evaluated by RT-PCR. As expected, when pUC β 128SV was transfected alone, only one transcript was amplified, which corresponds to the constitutively spliced β -globin mRNA (Fig. 9B, *lane 1*), whereas the three alternatively spliced transcripts (*transcripts 1–3*) were detected when pUC $\beta\Delta$ 128SV was used (Fig. 9B, *lane 7*). In the presence of increasing amounts of OTT3, the same amount of constitutive β -globin transcript was detected (Fig. 9B, *lanes 2* and *3*). Because the signal detected for the β -globin transcript was relatively strong, we needed to be sure that we were working in the linear range of the PCR amplification. We thus did a parallel control PCR amplification using 1/5 of the RT cDNA used in the assay depicted in Fig. 9B, and we found that the amount of amplified fragment was indeed proportional to the amount of RT cDNA used (data not shown). Thus OTT3 overexpression has no effect on the level of the β -globin transcripts detected. On the contrary, OTT3 overexpression has a drastic repressive effect on the level of the three alternatively spliced mRNAs expressed from pUC $\beta\Delta$ 128SV, as can be seen in Fig. 9B, *lanes 8* and *9*. This effect is not likely to be due to a defect in the export of these mRNAs because we did not find a specific accumulation of the corresponding mRNAs in the nuclei of the cells in the presence of OTT3 (data not shown). Similarly to OTT3 and as reported previously (54), the expression of EB2 induced a decrease in the amount of the alternatively spliced mRNAs expressed from pUC $\beta\Delta$ 128SV (Fig. 9B, *lanes 10* and *11*). However, it also induces the accumulation of unspliced mRNAs otherwise not detected in the cytoplasm of the cells. Again, there was no effect on the accumulation of the β -globin transcripts, although a minor band of unspliced mRNAs could be detected. But when F-OTT3 and F-EB2 were co-expressed, we observed a decrease in the amount of the alternatively spliced mRNAs detected, which confirmed the observation made with each protein alone. Moreover, we observed a decrease in the accumulation of the unspliced mRNAs induced by EB2. This is probably due to an inhibition of the function of EB2 via its interaction with overexpressed F-OTT3 that may result in EB2 being trap in nonfunctional complexes.

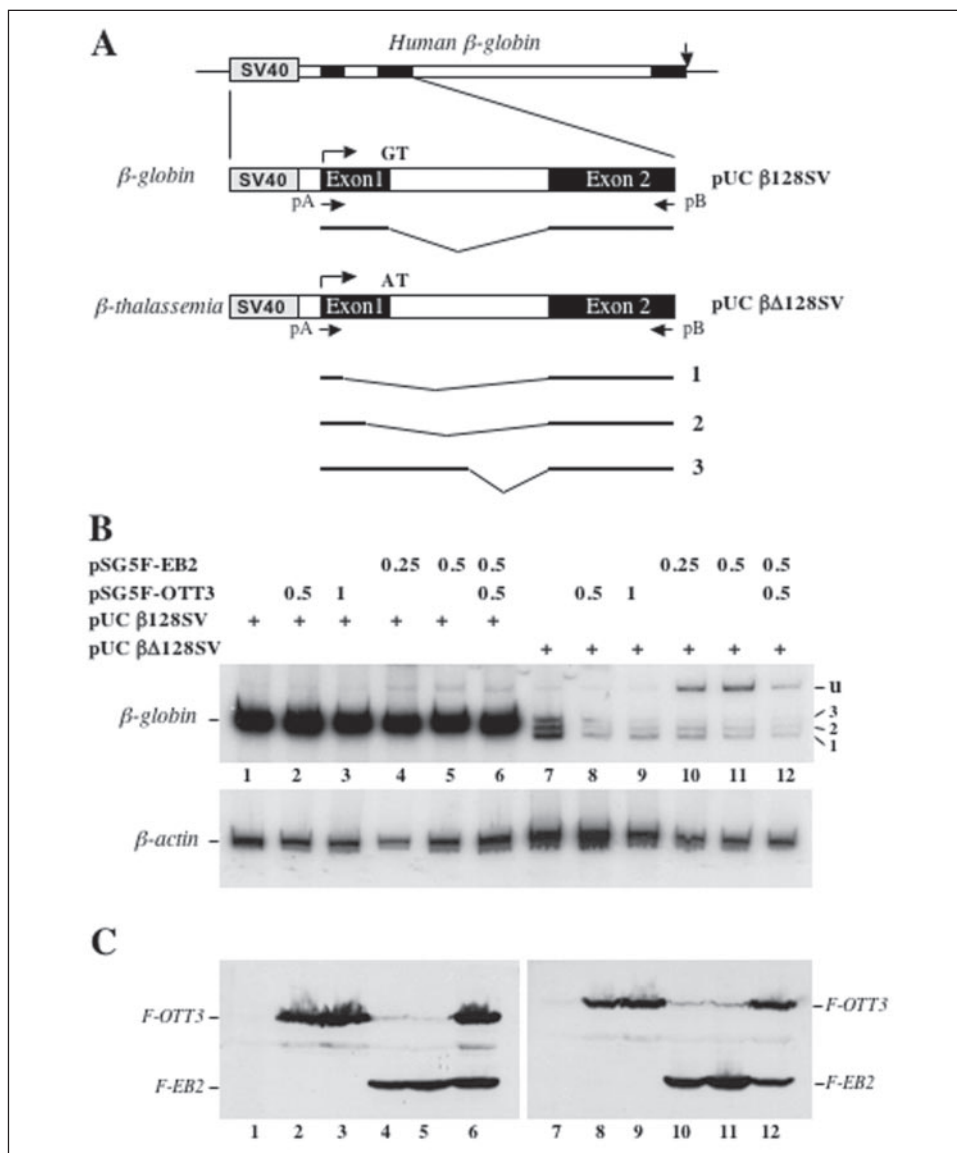
In conclusion, these results suggest that OTT3 acts at a post-transcriptional level by specifically repressing the accumulation of alternatively spliced β -thalassemia mRNAs without affecting the constitutively spliced β -globin mRNAs.

DISCUSSION

A yeast two-hybrid screen, using the EBV EB2 mRNA export factor as a bait, enabled us to characterize a novel protein, called OTT3, related to the *Drosophila* Spen (Split-end) family of proteins. Spen proteins vary widely in size (90–600 kDa) but are characterized by a conserved C-terminal structural domain called SPOC and the presence of three RRM at the N terminus of the proteins. Two proteins of the Spen family that occur in man have been described previously, SHARP (36) and OTT1/

FIGURE 9. OTT3 specifically represses accumulation of the alternatively spliced β -thalassemia transcripts but does not affect accumulation of the constitutively spliced β -globin transcript.

A, schematic representation of pUC β 128SV and pUC Δ 128SV reporter plasmids. The various mRNA species generated from these plasmids are represented. Arrows depicted as pA and pB represent the primers used for PCR amplification of the various reverse-transcribed mRNA. B, cytoplasmic RNA, extracted from the HeLa cells transfected with the reporter and expression plasmids as indicated in the figure, were reverse-transcribed using oligo(dT)₁₂₋₁₈ as a primer and then PCR-amplified in the presence of 1 μ Ci of [³²P]dCTP with the pA and pB primers depicted in A. The PCR-amplified fragments were separated on a 5% polyacrylamide gel and revealed by autoradiography. As a control, the endogenous actin mRNA was PCR-amplified from the same samples. The content of the alternatively spliced transcripts labeled 1–3 are depicted in A. u indicates the unspliced mRNA. C, Western blot analysis of F-OTT3 and F-EB2 proteins. Proteins extract from HeLa cells, transfected as indicated in B, were separated on an 8% SDS-PAGE, transferred to nitrocellulose, and incubated with an anti-FLAG antibody.



RBM15 (37, 38). SHARP, a large protein of 3664 residues, is a transcriptional repressor that can be recruited to its target promoters by interaction with nuclear receptors (36) or RBP- κ (43). Interaction domains with these proteins have been localized to a region of SHARP that is not conserved in either OTT1 or OTT3. The SPOC domain of SHARP has been shown to be responsible for the transcriptional repression via recruitment of the SMRT and N-CoR co-repressors (36). OTT1 is a much smaller protein (957 residues) of unknown function that was identified as a fusion protein with MAL, as a result of a recurrent t(1,22)(p13;q13) translocation exclusively associated with infant acute megakaryoblastic leukemia. From its size (889 residues) and the nature of its SPOC domain, OTT3 appears to be more closely related to OTT1 than to SHARP.

By Northern blot analysis, we found that the *OTT3* gene is transcribed as two mRNA species of 7.5 and 3.5 kb. Two mRNAs, respectively, 8.2 and 3.9 kb in size were also detected for *OTT1* (37), but although the 8.2-kb mRNA was found to be the most represented species in the case of *OTT1*, for *OTT3*, the most represented species is the smaller 3.5-kb mRNA. Similar to the 8.2-kb OTT1 mRNA, the 7.5-kb OTT3 mRNA contains a very large untranslated 3'-region as can be

deduced from several sequences deposited in the GenBankTM data bank under the name RBM15B. From the sequence of the cDNA clone IMAGE:3051463, it appears that the 3.5-kb mRNA species is generated by the use of a polyadenylation site proximal to that used for the 7.5-kb mRNA. There is no consensus cleavage/polyadenylation signal upstream of the poly(A) track found in this cDNA. However, the sequence, AGTAAA, located 24 nucleotides upstream, may serve as such a signal. The consequence of the absence of consensus cleavage/polyadenylation signal in the 3.5-kb mRNA may be an altered efficiency of cleavage/polyadenylation at this site, which would explain the use of a distal polyadenylation signal such as found in the 7.5-kb mRNA. The sequence of a murine RBM15B homologous mRNA has also been deposited in the GenBankTM data bank. Most interestingly, this mRNA also appears to be polyadenylated at a site devoid of a consensus polyadenylation signal, and again, an AGTAAA sequence is found upstream of the poly(A) track.

By using a monoclonal antibody generated against a recombinant OTT3 protein, we were able to detect a protein of 120 kDa in all cell types tested, corresponding with the ubiquitous expression of the mRNA. However, although the mAb detected the protein expressed

EBV mRNA Export Factor EB2 Interacts with Spen Proteins

from a plasmid very efficiently in a Western blot, the endogenous protein could only be detected following an immunoprecipitation step, to concentrate the protein. This suggests that the constitutive amount of OTT3 in the cells is not very high. Although we could not detect the endogenous protein in immunofluorescence experiments, the protein expressed from a plasmid was easily detected in the cell nucleus where it co-localized with the EB2 protein.

Interaction between OTT3 and EB2 was further demonstrated by *in vivo* co-immunoprecipitation assays and GST pull-down experiments using a fusion between the GST protein and the SPOC domain of OTT3. The results from GST pull-down experiments suggest that the interaction is likely to be direct. Furthermore, there is evidence that the interaction is independent of both proteins binding to RNA as follows: (i) EB2, which has its RNA-binding domain deleted, still co-immunoprecipitates with OTT3 *in vivo*; (ii) the RRM of OTT3 are not required for the interaction with EB2 because the GST pull-down experiments showed that the SPOC domain of OTT3 is sufficient for the interaction with EB2.

As the interaction between OTT3 and EB2 is via the SPOC domain and as this domain is conserved in the two other members of the family described in man, we were prompted to test a potential interaction between EB2 and the SPOC domains of both SHARP and OTT1. Although EB2 was indeed found to interact with both OTT1 and SHARP SPOC domains, surprisingly, the EB2 domains involved are different in the case of OTT3. Deletion of the 51 N-terminal residues of EB2 is highly deleterious for the interaction with SPOC-OTT3, whereas the interaction with both SPOC-OTT1 and SPOC-SHARP is only slightly affected. The domain of EB2 important for interaction with SPOC-OTT1, and SPOC-SHARP appears to be present in the C-terminal half of the protein. It was not possible to characterize the interaction region further however, as all deletion mutants generated in the C-terminal part of the protein appear to have a strong deleterious effect on the overall function of the protein. In particular, when expressed in HeLa cells, these mutated proteins are abnormally localized in the form of very large foci or aggregates in the nuclei.⁸ That different domains of EB2 interact with the SPOC domains of the three human Spen proteins albeit via different regions suggests in turn that the EB2-binding regions within the SPOC domains of OTT3, OTT1, and SHARP also differ. Despite these differences, it is interesting to note that the interaction of EB2 with all the members of the Spen family is a conserved property of the protein and is thus likely to be important for EBV biology.

A common function of the SPOC domains has been suggested to be their capacity to act as a transcriptional repression module that recruits the universal co-repressors, SMRT and NCoR. This has been demonstrated in the case of the SHARP SPOC domain, and the authors (39) responsible for the elucidation of its crystal structure suggested that it is also the case for the other SPOC domains. In effect, the residues found on the molecular surface of the SPOC domain are essentially conserved across the whole family. Furthermore, a structure-based mutational analysis indicated that a conserved positively charged patch on the surface of the SPOC module was responsible for the interaction between SHARP and SMRT/NCoR. Although OTT3 was not yet characterized at the time and thus could not be included in the comparative alignment, its SPOC domain is closely related to that of OTT1, which was predicted to also interact with SMRT/N-CoR. Our results, however, suggest that of the three human Spen proteins, only SHARP interacts efficiently *in vitro* with SMRT and strongly represses transcription *in vivo* via its SPOC domain. This suggests that there are other residues in the SPOC domain, outside the conserved patch, which probably play an

essential role in the interaction with SMRT. It is interesting to note that although the SPOC domain of OTT1 and OTT3 displays the closest homologies and are both divergent from SPOC SHARP in their capacity to interact with SMRT, OTT1 shares with SHARP the capacity to interact with the C terminus of EB2, whereas OTT3 interacts with the N terminus of the protein; each of the three SPOC domains display specific protein-protein interaction surfaces.

The functions of both OTT1 and OTT3 are as yet unknown. Neither is likely to be involved in promoter-dependent transcriptional repression in the same way as SHARP, because they both lack the SHARP domains responsible for interaction with nuclear receptors or RBP-J κ that can target SHARP to specific promoters. Moreover, their SPOC domains have only faint repressing activity. There is evidence suggesting that the Spen proteins are involved in mRNA biogenesis as follows. (i) All three contain several RNA-binding regions. (ii) Both OTT1 and SHARP have been found in the purified spliceosome (OTT3 had not been described at the time) (35). In accordance with this potential role in mRNA biogenesis, we found in transitory transfection experiments that OTT3 appears to have a strong inhibitory effect on the cytoplasmic accumulation of β -thalassemia alternatively spliced mRNA, although it has no effect on the accumulation of the constitutively spliced β -globin transcript. Because both genes are expressed from the same SV40 promoter and only differ by a single mutation, the observed effect is clearly not due to an effect on transcription. Moreover, the fact that we did not find a retention of these mRNA in the nuclei of the cells suggests that OTT3 is likely to have a direct role in the inhibition of splicing using the "illicit" 5'-splice sites revealed in the β -thalassemia gene following mutation of the constitutive 5'-splice site of the β -globin gene. Alternatively, OTT3 may function by recognition of this illicit splicing. In both cases, either because the mRNA has not been spliced or because OTT3 has marked this mRNA as inadequately spliced, the mRNA would then be further degraded by the nuclear exosome (55). Whether the observation of an inhibitory effect of OTT3 on the accumulation of the β -thalassemia transcripts can be extended to other systems in which alternative or illicit splicing occurs will now have to be determined.

In this model, by targeting OTT3, EB2 could specifically target mRNAs generated by the use of nonconstitutive 5'-splice sites and induce their export as unspliced mRNA. We are currently investigating the importance of OTT3, OTT1, and SHARP for the biology of EBV and more precisely the function of EB2 in the productive cycle of the virus. Given the structural homology between OTT3 and OTT1, these proteins may have redundant functions in the cells. However, because EB2 interacts with OTT3 and OTT1 via distinct domains, it has proved difficult to generate EB2 mutants altered for interaction with both proteins without affecting other functional domains of the protein. The use of RNA interference in order to silence expression of OTT3, OTT1, and SHARP independently or together should allow us to determine both the cellular function of these proteins and their role in the productive cycle of EBV.

In conclusion, we have characterized a novel protein of the Spen family, huOTT3, and we have demonstrated that it, together with the other members of the Spen family known in man, interacts with the EBV mRNA export factor EB2. This interaction takes place via the SPOC domains of the Spen proteins. Although the SPOC domains were described previously as being structurally conserved with putative identical functions, our results suggest that they exhibit different specificities in their interactions with the viral factor EB2 and the SMRT co-repressor protein. This in turn suggests that there are distinct functions for the SPOC domains of the various Spen proteins. Finally and most importantly, we have demonstrated a new potential function for at least one protein of the Spen family, OTT3, in the regulation of alternative or illicit splicing.

⁸ E. Hiriart, M. Buisson, A. Sergeant, and E. Manet, unpublished data.

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