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The neurofibromatosis type I messenger RNA undergoes base-modification RNA editing

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ABSTRACT

A functional mooring sequence, known to be required for apolipoprotein B (apoB) mRNA editing, exists in the mRNA encoding the neurofibromatosis type I (NF1) tumor suppressor. Editing of NF1 mRNA modifies cytidine in an arginine codon (CGA) at nucleotide 2914 to a uridine (UGA), creating an in frame translation stop codon. NF1 editing occurs in normal tissue but was several-fold higher in tumors. *In vitro* editing and transfection assays demonstrated that apoB and NF1 RNA editing will take place in both neural tumor and hepatoma cells. Unlike apoB, NF1 editing did not demonstrate dependence on rate-limiting quantities of APOBEC-1 (the apoB editing catalytic subunit) suggesting that different *trans*-acting factors may be involved in the two editing processes.

INTRODUCTION

RNA editing has been described in diverse eukaryotic organisms and can be mechanistically categorized as either RNA strand scission and ligation (insertion editing) or base-modification editing. (1). There are five current examples of mammalian cellular RNA editing. Four clearly belong to the base modification category of editing yet no common mechanism has emerged.

Apolipoprotein B (apoB) mRNA editing involves cytidine to uridine conversion at nucleotide 6666 and creates an in-frame translation stop codon (UAA) from a glutamine codon (CAA) (2,3). Site-specific editing of apoB mRNA is mediated by a cytidine deaminase, APOBEC-1 (4–6) whose activity on RNA is dependent upon its assembly with one or more auxiliary proteins (4,5,7) as an editosome (8).

Site-directed mutagenesis has defined the apoB mRNA editing site. It consists of a tripartite, 21 nucleotide motif containing a spacer sequence element and a mooring sequence, both 3′ of the cytidine to be edited, and a regulator element immediately 5′ of the cytidine (9–12). The mooring sequence is the only element within the tripartite motif which is necessary and sufficient for site-specific editing (9,10,13). Recognition of the mooring sequence during editosome assembly has been largely attributed to RNA-binding proteins of 40–66 kDa (8,14,15), although APOBEC-1 has a non-specific and low affinity RNA-binding capacity (16,17).

Editing involving adenosine deamination has been demonstrated in mRNA encoding receptor subunits (GluR-B) of the AMPA subtype of glutamate-gated ion channels which mediate fast excitatory neurotransmission (18). Adenosine is deaminated to form inosine in the context of a CAG glutamine (Q) codon (19–21). The Q/R amino acid substitution occurs within the second hydrophobic segment of the protein and is essential for reducing Ca^{2+} permeability of ion channels containing the GluR-B subunit. Editing site recognition may be mediated through RNA secondary structure formed between unique RNA sequences flanking the editing site in exon 11 and an exon-complementary sequence (ECS) within the downstream intron (22,23). A double-stranded RNA adenosine deaminase, believed to be responsible for GluR-B mRNA editing, has been isolated from mammalian tissues and characterized (24,25).

Editing of the Wilms' tumor suppressor gene product (WT1) mRNA has been observed in both human and rat tissues and appears to involve amidation of uridine to cytidine at nucleotide position 839 (26). Edited WT1 [containing a proline (CCC) substituted for leucine (CUC)] was less effective in regulating transcription in transfected cells from the early growth response promoter (26), suggesting a potential role for editing in the pathogenesis of Wilms' tumor.

Editing of the mRNA encoding α-galactosidase, the lysosomal enzyme deficient in Fabry's disease, occurs at nucleotide position 1187 and involves a uridine to adenosine transversion resulting in a Phe to Tyr amino acid substitution (27). The mechanism for this form of editing appears not to be base modification and its biological consequence is uncertain at this time.

In addition to mRNA editing, $tRNA^{Asp}$ editing has been described in rodents and marsupials. The majority of rat liver cytoplasmic $tRNA^{Asp}$ is edited at two sites adjacent to the anticodon loop involving a C→U and U→C modification at positions 32 and 33 respectively (28). Up to 50% of marsupial mitochondrial $tRNA^{Asp}$ is also edited but modification occurs in this instance within the anticodon and involves a GCC to GUC conversion (29). The mechanism(s) and factors remain to be

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determined in what appears to be both deamination and amidation processes.

The number of examples of RNA editing in mammals and the diversity of the biological systems affected suggest that editing may be a common mechanism for regulating gene expression. An editing site within the mRNA encoding the neurofibromatosis type I (NF1) gene product, neurofibromin, has been identified which is predicted to result in loss of tumor suppressor gene product function due to the introduction of a premature, in-frame translation stop codon.

MATERIALS AND METHODS

Tissues, cell culture and RNA preparation

Tumor tissues were obtained from the University of Rochester Medical Center and from Medical Centers throughout the United States. In every case the tissue was frozen without delay and either shipped on dry ice or stored at –80°C until the time of RNA preparation. All sample collection was approved by the Institutional Review Board at the respective site of origin. All patients diagnosed with NF1 conformed to the standard diagnostic criteria prescribed by the National Institutes of Health Neurofibromatosis Consensus Development Conference (30). A172, NFRL91, NGP, K562 and ST-88-14 cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. 14942 lymphoblastoid cells were cultured in RPMI 1640 with 15% fetal bovine serum and U373 cells were cultured in DMEM (high glucose) supplemented with 10% fetal bovine serum. McArdle RH7777 were obtained from the ATCC (Rockville, MD) and grown in DMEM containing 10% fetal bovine serum and 10% horse serum. Cells were subcultured when they reached 80% confluence.

RNA was prepared from cells by washing the monolayers three times with phosphate buffered saline (PBS) followed by lysis according to the procedure of Chomczynski and Sacchi (31) or in the Tri-Reagent (MRC) according to the manufacturer's recommendation. RNAs were digested with RQ-DNase I (Promega) for 30 min followed by an additional 30 min digestion with an appropriate restriction enzyme having a recognition site between the PCR primer annealing sites. RNAs were phenol extracted, precipitated and quantified by spectrophotometry.

Peripheral blood lymphocytes were isolated from whole blood by centrifugation (1000 *g*, 20 min) to separate the cellular components from the serum. The overlying serum was removed and the buffy coat collected by aspiration. The cells were then washed three times in 45 ml ammonium chloride and sodium bicarbonate. RNA was prepared from the isolated cells as described above.

Preparation of first strand cDNA and polymerase chain reactions

For NF1 analyses, first strand cDNA was prepared from 1 µg of total cellular RNA using 100 pg of random hexamers/µg of reaction (Amersham) and AMV reverse transcriptase (Promega) with the manufacturer's recommended buffers and conditions. For apoB analyses, first strand cDNA was primed on 1 µg total cellular RNA with 1 µg oligo-dT using AMV reverse transcriptase (Promega) with the buffers and conditions recommended by the manufacturer.

PCR was carried out on 9.0 µl of each reverse transcription reaction in a final volume of 100 µl containing 0.5 µM each of NF1A and NF1D as amplimer pairs for the endogenous NF1 transcript or SP6 and T7 promoter-complementary amplimer pairs for the NF1 transcript expressed from transfected pRc/CMV vector (Invitrogen). Polymerase chain reactions (PCR) were carried out in the presence of 2.5 mM $MgCl_2$ using the manufacturer's buffers and 1.5 U *Taq* DNA polymerase (Promega) for 30 cycles. Thermal cycle conditions for NF1 amplification were: 94°C for 1 min, 60°C for 1 min and 72°C for 2 min.

PCR amplification of the endogenous rat apoB sequences encompassing the editing site and those from the transfected human apoB cDNA was performed on first strand cDNA using amplimers ND1/ND2 (32) and SP6/T7 respectively. PCR was performed with *Taq* DNA Polymerase (Promega) in the presence of 2.5 mM $MgCl_2$ according to the manufacturer's recommendations. Thermal cycling conditions for apoB were: 1 cycle at 94°C for 2 min; 5 cycles at 94°C for 45 s, 52°C for 1.5 min, 72°C for 1.5 min; and 30 cycles at 94°C for 45 s, 55°C for 1 min, 72°C for 1 min.

PCR products were gel isolated (Qiaex, Qiagen, Inc.) and quantified by comparison to a DNA mass ladder (Gibco) following gel electrophoresis. Through the direct sequencing of individually cloned PCR products we have determined the error frequency to be 0.21% or 1 base error every 476 bases resulting from reverse transcriptase, PCR or dideoxy sequencing nucleotide mis-incorporation (33).

Poisoned primer-extension assay for RNA editing

Gel isolated, deoxy oligonucleotides used for primer extension of apoB and NF1 PCR products (DD3 and SK1, respectively) were 5′ end-labeled with [γ-^{32}P]ATP (NEN) using T4 polynucleotide kinase (US Biochemical) according to the manufacturer's protocol. Primer-extension assays on PCR products from the endogenous genes' transcripts were carried out using 50 pg to 10 ng of input PCR product template DNA and 40 fmol of end-labeled primers in the presence of 200 µM each dATP, dCTP, TTP and 800 µM 2′3′ ddGTP as described previously (9,10). The reaction products were resolved under denaturing conditions on 10% acrylamide gels containing 7 M urea, visualized by autoradiography and quantified by PhosporImager scanning (Molecular Dynamics; Model 425E) or by liquid scintillation counting of excised first and second stop gel bands. For quantification, assays were performed in triplicate on the indicated number (n) of independent RNA isolations.

Poisoned primer-extension assays of *in vitro* editing activity were performed directly on RNAs isolated from the *in vitro* reactions using ^{32}P end-labeled DD3 and SK1 for apoB and NF1 RNA respectively and the extension products analyzed as described above. Editing levels were quantified as described above.

Single strand conformation polymorphism (SSCP) assay for edited NF1 RNA

For SSCP analyses, a 64mer was amplified by the PCR from 9 µl of a reverse transcription reaction using 1.0 U *Taq* polymerase (Promega), SK1 and SK4 as amplimer pairs, 2.0 µCi of [α-^{32}P]dATP (3,000 Ci/mmol) (NEN) and buffers provided by Promega. Thermal cycling conditions were: 94°C for 1 min, 66°C for 1 min and 72°C for 30 s for 30 cycles. One µl of each reaction mixture was diluted 1:100 in 0.1% sodium dodecylsulfate, 10 mM EDTA followed by a 1:1 dilution in 95% formamide, 20 mM EDTA and heating to 90°C for 3 min. Comformers were resolved on 12% acrylamide gels, dried and autoradiographed.

Extract preparations and *in vitro* editing

The rat liver hepatoma cell line, McArdle, and NGP cells were grown to 80% confluence in forty 100 mm dishes. Extracts were prepared by rinsing the cells once with PBS followed by scraping into 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM dithiothreitol, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 20 U/ml soybean trypsin inhibitor (Sigma Chemical Co.), 0.5 μg/ml aprotinin (Sigma) and 0.5 μg/ml leupeptin (Sigma). Cells were cleared by centrifugation (300 *g*, 10 min) and resuspended in five packed cell volumes of the buffer described above (diluted to one tenth strength) and allowed to swell on ice for 30–40 min. Cells were centrifuged (2500 *g*, 10 min), resuspended in 2 packed cell volumes in the same buffer and sheared sequentially through 18, 20 and 22 gauge needles at high pressure. Extracts were brought to 200 mM KCl, allowed to incubate on ice for 15 min and cleared at 13 000 *g*, 30 min. All procedures were performed at 4°C with sterile, diethyl pyrocarbonate-treated buffers. Extracts were stored at –20°C.

In vitro editing assays were performed as described previously (9,10) using 60 μg of extract protein and 20 fmol of either apoB RNA substrate (448 nucleotides corresponding to nucleotides 6413–6860 of human apoB cDNA, plus 50 nucleotides from pGEM4 polylinker sequence) or NF1 RNA substrate (472 nucleotides corresponding to nucleotides 2689–3161 of human NF1 cDNA, plus 84 nucleotides from pcDNAIII polylinker sequence). The 498 nucleotide apoB RNA transcript is composed of 75% AT while the 556 nucleotide NF1 RNA transcript is 53% AT. *In vitro* [^{3}H]ATP (NEN) labeled transcripts were synthesized from *Kpn*I linearized apoB and *Xba*I linearized NF1 plasmids using T7 RNA polymerase (Promega) with the manufacturer's buffers and conditions.

Preparation of transfection constructs and transfected cell lines

APOBEC-1 (4) cDNA was produced from oligo (dT)-primed, rat small intestine, total RNA using AMV reverse transcriptase (Promega) and amplified with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's recommendations in a PCR reaction using REPR-5′ and REPR-3′ as amplimers involving 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The PCR product (extending from nucleotides 36 to 740 on APOBEC-1 cDNA and containing the ATG at nucleotide 42) was subcloned into pSP73 (Promega) through the *Pst*I and *Eco*RI restriction sites in the amplimers, verified by dideoxy DNA sequencing and subcloned into the *Hin*dIII and *Eco*RV sites of pRc/CMV.

The wild type human apoB transfection construct was generated by subcloning the human apoB cassette from pRSA13 (3) as a *Hin*dIII–*Kpn*I fragment into *Hin*dIII–*Xba*I linearized pRc/CMV. *Kpn*I and *Xba*I restriction ends were made blunt by the use of T4 DNA polymerase (Gibco) and the Klenow fragment of DNA polymerase I (Promega) according to the manufacturer's recommendations. The expressed transcript corresponds to nucleotides 6413 to 6860 of apoB cDNA.

The wild-type NF1 transfection construct was generated by PCR amplification of the human cDNA insert from FB5D (NF1 cDNA plasmid, kindly provided by Dr Francis S. Collins) using GS3 and GS4 as amplimer pairs and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The NF1 PCR product corresponding to human NF1 sequences from nucleotides 2689 to 3169 was blunt-end ligated into the *Eco*RV site of pRc/CMV and selected by cloning and dideoxy sequencing (34) for the sense strand to be oriented relative to the T7 promoter.

McArdle, NGP and A172 cell lines were transfected with the indicated constructs according to the method of Chen and Okayama (35). 48–72 h post DNA addition, cells were either harvested for RNA isolation (transient transfections) or placed under selection in 300–500 μg/ml G418 (Gibco) for the establishment of stable transfected cells.

Deoxy oligonucleotides use in this study

DD3	5′-AATCATGTAAATCATAACTATCTTTAATATACTGA
GS3	5′-CTCCCCGGGGTTCCTTGTTCTCAGTGGG
GS4	5′-CTCTCTAGAATGGCACTTCCTACTGCAC
NF1GA	5′-AGGTTACCACAAGGATCTCCAGACA
NF1GD	5′-CCTGCGTGCTGCATCAAAGTTGCTT
ND1	5′-ATCTGACTGGGAGAGACAAGTAG
ND2	5′-GTTCTTTTTAAGTCCTGTGCATC
REPR-5′	5′-CTCCTGCAGCAAGATGAGTTCCGAGACAG 3′
REPR-3′	5′-CTCGAATTCCCAGAAGTCATTTCAACCCTG 3′
SK1	5′-CATGTTGCCAATCAGAGGATGTG
SK4	5′-TATCTACAAAAACTCCTGGATCC
SP6	5′-GCTCTAGCATTTAGGTGACACTATAG 3′
T7	5′-TAATACGACTCACTATAGGG 3′

RESULTS

Mutational analyses have shown that the mooring sequence functions in directing editing of appropriately positioned 5′ cytidines in a variety of RNA contexts (10,12,13,32,36,37). These data suggest the possibility of mooring sequence-dependent RNA editing in RNAs other than apoB providing that certain sequence requirements are met and the appropriate *trans*-acting factors are present. To this end, we have evaluated the possibility of using the mooring sequence as a means of screening for potential editing substrates. In cDNA, the 5′ end of the mooring sequence is a *Bcl*I restriction endonuclease site. We have utilized the MAP program of the University of Wisconsin Genetics Computer Group (38) to evaluate the occurrence of *Bcl*I recognition sequences residing 3–5 bases 3′ of a cytidine (or thymidine) specifically within the NF1 cDNA.

A unique site having a high degree of homology with the 21 nucleotide apoB editing site was identified at nucleotide position 2909–2930 (using nucleotide coordinates taken from GenBank accession number M38107) (Fig. 1) within the mRNA encoded by the tumor suppressor gene responsible for von Recklinghausen neurofibromatosis or neurofibromatosis type I (NF1). Four additional *Bcl*I sites were identified but none had the other requisite features of an apoB-like editing site. The sequences within this region of the NF1 mRNA which correspond to the mooring sequence, spacer and regulator element are indicated in Figure 1. The rat NF1 homolog does not contain an appropriate mooring sequence motif (Fig. 1). The six nucleotide stretch separating the presumptive edited cytidine at nucleotide position 2914 (C2914) from the 5′ end of the human NF1 mooring sequence is two nucleotides longer than would be ideal for apoB RNA editing and contains two guanidine residues which would be tolerated by the apoB editing apparatus but not preferred (10). Both the mooring sequence and the regulator element of the NF1 site are comprised of sequences which should support editing based on site-directed mutagenesis of apoB RNA (10).

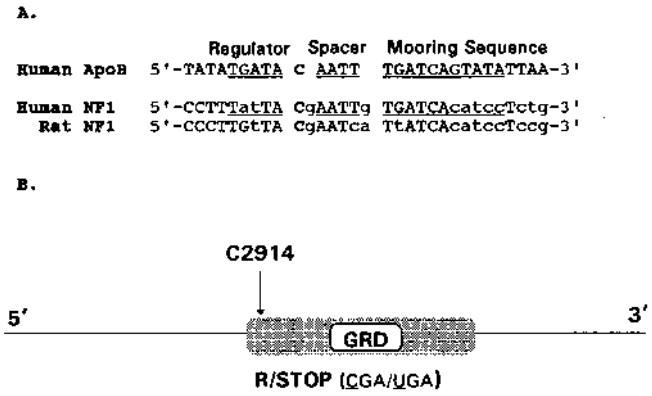

Figure 1. Sequence comparison between the apoB mRNA editing site and its NF1 homolog. (**A**) The apoB mRNA editing site is a tripartite motif consisting of a regulator and spacer element 5′ and 3′ respectively of the cytidine to be edited and a 3′ mooring sequence (shown underlined on the apoB mRNA sequence). Homologs to these sequences in human NF1 mRNA are underlined and shown below the corresponding apoB sequences. (**B**) The target of NF1 mRNA editing, C2914, is located at the 5′ end of the GAP related domain (GRD). C→U editing converts an arginine codon (R) to a translation stop codon and has the potential to encode a truncated polypeptide lacking the GRD.

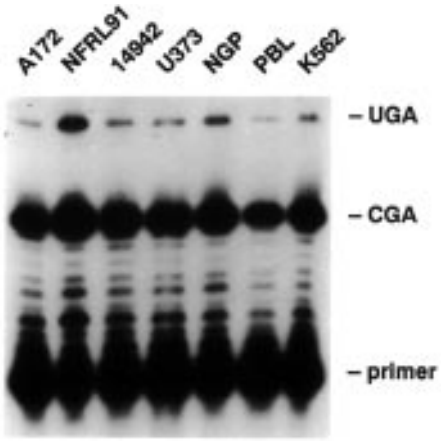

Figure 2. Identification of NF1 mRNA editing at C2914. RNA isolated from various cell lines and from circulating leukocytes was assayed for NF1 mRNA editing by poisoned primer-extension assay and quantified as described in Materials and Methods. Bands corresponding to unextended primer (primer), primer-extension products from unedited NF1 RNA (CGA) and primer-extension products from edited NF1 RNA (UGA) are indicated to the right. The cell lines assayed are indicated above each lane and correspond to: A172 (glioblastoma), NFRL91 (neurofibrosarcoma), 14942 (lymphoblastoid), U373 (astrocytoma), NGP (neuroblastoma) and K562 (chronic myelogenous leukemia). Peripheral blood lymphocytes (PBL) from a non-NF1 individual were also assayed. Editing efficiency for A172, NFRL91, 14942, U373, NGP, K562 and PBL was 2.4, 2.0, 1.5, 1.2, 3.3, 1.5 and 1.9 percent respectively (± 0.07–0.2 S.D., n = 3).

Figure 3. Confirmation of NF1 mRNA editing by SSCP analysis. A 64 bp region of the NF1 cDNA was amplified by the PCR and assayed for single-strand conformation polymorphisms as described in Materials and Methods. The two bands corresponding to unedited PCR products are indicated by arrowheads to the right while an additional band arising from the edited sequence is indicated by the arrow to the left. The source of the control unedited sequence is the NF1 cDNA clone FB5D which is known to carry a C at position 2914. The cell lines assayed are indicated above each lane and correspond to: A172 (glioblastoma), NFRL91 and ST88-14 (neurofibrosarcoma), NGP (neuroblastoma), K562 (chronic myelogenous leukemia) and 14942 (lymphoblastoid). RNA from an NF1 tumor was also assayed (tumor).

Sequence context has a profound effect on the efficiency of utilization of the apoB editing cassette. AT-rich distal flanking sequences are preferred over GC-rich sequences (9,13). The sequence to either side of the presumptive NF1 editing site is 53% AT compared to 75% AT in the flanking sequence of the apoB editing site. Considering this and the suboptimal spacer region, we predicted that although human NF1 has the potential to be an editing site it may only function at 20–40% of the efficiency of the apoB editing site (if both were assayed in the context of liver cells or liver cell extracts).

To evaluate the potential of human NF1 mRNA editing, total cellular RNA was isolated from cell lines representing tumor types which commonly occur within NF1, namely, glioblastoma, astrocytoma, oligodendroglioma and neurofibrosarcoma, as well as neuroblastoma, chronic myelogenous leukemia, transformed lymphoblasts and normal peripheral blood leukocytes (Fig. 2). First strand cDNA was synthesized and NF1-specific sequence containing the presumptive editing site was amplified by the polymerase chain reaction (PCR) using GS3 and GS4 amplimers as described in Materials and Methods. Editing of C2914 was evaluated by a poisoned primer-extension assay (primer extension in the presence of ddGTP) similar to that used to assay apoB mRNA editing (30) and quantified as described in Materials and Methods. In each lane of the autoradiograph shown in Figure 2, three major bands were apparent corresponding to primer, the first stop primer-extension product arising from unedited NF1 mRNA (CGA) and the second stop primer-extension product arising from edited NF1 RNA (UGA). The data indicate that C2914 was edited at low levels in peripheral blood leukocytes (PBL) isolated from unrelated, non-NF1 volunteers (1.9% editing ± 0.2 S.D., n = 12). NF1 RNA was also edited in tumor cell lines with varying efficiency (1–4% ± 0.07–0.2% S.D., n = 3).

No read-through was observed with the human NF1 cDNA clone, FB5D, which has a C at position 2914 (data not shown but see Fig. 3). This control for primer-extension read-through, a potential source of an editing false positive, indicates that ddGTP chain termination at the upstream cytidine (nucleotide position 2906, C2906) was the result of editing of C2914. The absence of additional read-through from C2906 suggests that this upstream cytidine was not edited. Moreover, editing could not be demonstrated on rat NF1 mRNA isolated from McArdle cells (data not shown), presumably due to the degenerate mooring sequence in rat NF1 mRNA (Fig. 1). Taken together, these findings suggest that mooring sequence-dependent, base-modification editing has occurred at C2914 of human NF1 mRNA.

Single-strand conformation polymorphism (SSCP) assays were performed as a non-quantitative means of confirming editing of NF1 RNA through the detection of editing-dependent conformers. A 64 bp region surrounding C2914 was amplified by

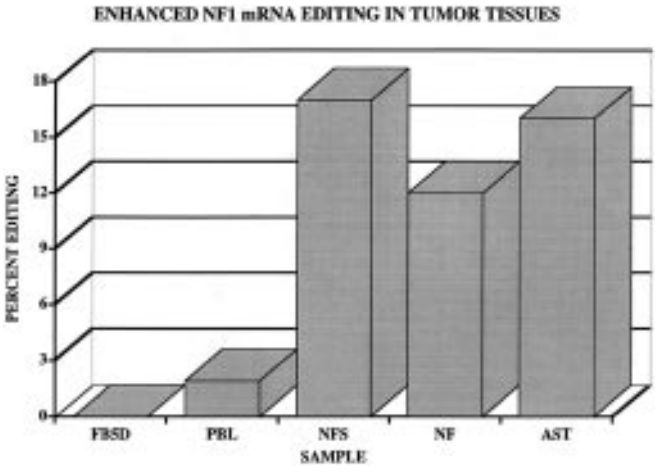

Figure 4. Comparison of NF1 mRNA editing efficiency in normal and tumor tissues. RNA isolated from control peripheral blood leukocytes (PBL) and from three tumors obtained from three unrelated NF1 patients was assayed for mRNA editing by poisoned primer-extension as described in Materials and Methods. An elevated level of NF1 mRNA editing relative to control was observed in every tumor analyzed. FB5D (cDNA clone with a C at position 2914), NFS (neurofibrosarcoma), NF (neurofibroma), and AST (astrocytoma). Values of the histograms correspond to the percent editing seen in each tumor and represent an average of three independent assays. Standard deviation for PBL was ±0.5% S.D. and was ≤ 1.4% S.D. for NFS, NF and AST.

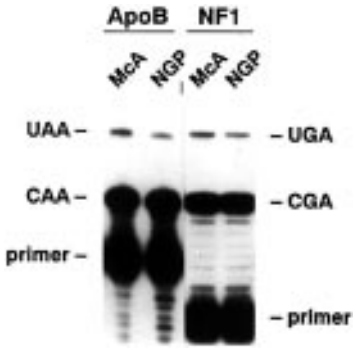

Figure 5. *In vitro* editing of apoB and NF1 RNA. *In vitro* transcripts of apoB and NF1 RNA containing the respective editing sites at C6666 and C2914 (respectively) were incubated with McArdle (McA) or NGP cell extracts under *in vitro* editing conditions and subsequently isolated and assayed by poisoned primer-extension for base-modification editing as described in Materials and Methods. Bands corresponding to unextended primer (primer), primer-extension products from unedited RNA (CAA for apoB and CGA for NF1) and primer-extension products from edited RNA (UAA for apoB and UGA for NF1) are indicated.

PCR and subjected to SSCP analysis as described in Materials and Methods. Electrophoretic analysis revealed differences between the amplification products of cellular cDNA and those of the unedited control, FB5D cDNA (Fig. 3). Two conformers corresponding to unedited sequence were resolved from SSCP with FB5D PCR products and from PCR products of all indicated cell sources. In addition, a unique conformer was present among PCR products synthesized from RNA isolated from six out of seven cell lines which previously demonstrated NF1 RNA editing. These data, though not confirmatory in one case (NGP), do indicate sequence polymorphism in the RT–PCR products of NF1 RNA containing the editing site and strongly support the conclusion that NF1 RNA is edited from quantitative primer-extension assays. The results from primer-extension analyses and SSCP have been confirmed through cloning and sequencing of RT–PCR products (data not shown, but see Materials and Methods).

Regions of neurofibromin share homology with the catalytic domains of the mammalian GTPase activating protein (GAP) and the yeast 'inhibitor of ras' proteins (IRA1 and IRA2) (39,40). Functional homology of the neurofibromin GAP-related domain (GRD) to the yeast IRA proteins was demonstrated by complementation in yeast deficient for IRA function (41). This homology with mammalian and yeast GAPs suggests that neurofibromin plays a role in the ras signal transduction pathways. Editing of C2914 would change an arginine codon (CGA) to a translation stop codon (UGA), thereby truncating NF1 just N-terminal to its GRD encoding sequence. A functional loss of tumor suppressor activity could therefore be one consequence of NF1 RNA editing. To evaluate the extent to which editing might be occurring in tumor tissues, RNA was isolated from several tumor specimens from unrelated NF1 patients and assayed for NF1 mRNA editing. RNA isolated from each specimen demonstrated up to 8.5-fold higher levels of NF1 mRNA edited compared to normal, peripheral blood leukocytes (Fig. 4). The data also demonstrated that editing in the NF1 cell lines is significantly lower than that detected in RNA isolated from solid tumors.

In addition to the tripartite editing cassette, apoB mRNA editing requires a specialized, *trans*-acting cytidine deaminase (APOBEC-1). This enzyme acquires site-specific RNA editing activity in the context of an editosome (8) through its interactions with yet to be characterized, *trans*-acting, auxiliary proteins (4,5,7,16,17,42–44). Identification of NF1 mRNA C→U editing through mooring sequence homology suggests that the mechanism for NF1 editing may also involve APOBEC-1 and auxiliary proteins. We initially addressed this question *in vitro* by evaluating whether extracts from rat hepatoma cells (McArdle 7777) and a human neuroblastoma cell line (NGP) had the capacity to edit both apoB and NF1 RNA substrates.

Extracts from McArdle and NGP cells were obtained by a modification of the protocol used previously in the preparation of extracts from rat enterocytes (9). Twenty fmols of apoB or NF1 RNA substrate were subjected to a 3 h *in vitro* editing assay as described in Materials and Methods. The RNA substrates had approximately equivalent length and contained the editing site at approximately their mid point. Editing activity was observed in both extracts with either RNA substrate (Fig. 5), although for the same amount of input protein, editing was 3-fold more efficient on either apoB or NF1 RNA substrates in McArdle cell extracts. The data strongly support the possibility that all the *trans*-acting factors involved in editing apoB and NF1 are present in both cell types.

Several laboratories have shown that in adult liver and liver cell lines, the proportion of apoB mRNAs which are edited in a cellular population ('the editing efficiency') is determined by rate limiting quantities of APOBEC-1 (42–44). The auxiliary factors required for APOBEC-1 mRNA editing activity occur in a wide variety of cell types irrespective of the occurrence of APOBEC-1 or apoB mRNA (4,5,7). To evaluate these characteristics, editing activity on endogenously expressed NF1 was assayed in APOBEC-1-expressing, stably transfected, glioblastoma (A172) and

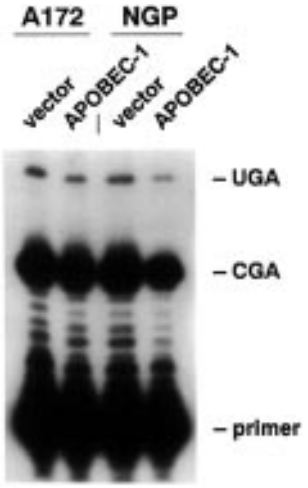

Figure 6. APOBEC-1-independent editing of NF1 mRNA in A172 and NGP cells. Editing of the endogenous NF1 genes' transcript was assayed and quantified as described in Materials and Methods on RNA isolated from stable transfectants of glioblastoma (A172) and neuroblastoma (NGP) cell lines carrying either the pRc/CMV vector alone (vector) or expressing APOBEC-1. Bands corresponding to unextended primer (primer), primer-extension products from unedited NF1 RNA (CGA) and primer-extension products from edited NF1 RNA (UGA) are indicated to the right. Editing efficiency for A172 and NGP vector alone transfected cells was 2.35% ± 0.21% S.D. and 3.3% ± 0.12% S.D. (n = 3) respectively and for A172 and NGP APOBEC-1 transfected cells was 2.30% ± 0.14% S.D. and 3.1% ± 0.15% S.D. (n = 3) respectively.

neuroblastoma (NGP) cells. Surprisingly, APOBEC-1 had little or no effect on editing of the endogenous NF1 mRNA relative to control cells transfected with a non-recombinant vector in either cell background (Fig. 6). The data suggest that although NF1 mRNA may share an RNA sequence motif with apoB RNA, the *trans*-acting factors involved in its editing may be distinct.

To further evaluate the occurrence of auxiliary factors which might support APOBEC-1 activity in neuroblastoma cells, stable transfected NGP cells (described above) and a stable transfected McArdle cell line expressing APOBEC-1 were assayed for their editing activity on apoB RNA. As expected, elevated levels of APOBEC-1 expression in transfected McArdle cells stimulated editing efficiency on the cells' endogenous apoB mRNA transcripts (Fig. 7). Phosphorimager quantification demonstrated that editing in control McArdle cells (16%) had increased to 30% (±1.5% S.D., n = 3) in this particular APOBEC-1-expressing cell line.

NGP cells do not express an endogenous apoB mRNA and therefore the occurrence of an apoB-like editing activity in the APOBEC-1, stable transfectants was assessed by transient expression of apoB RNA. RNA isolated from NGP cells 48 h after transient transfection demonstrated a very low but reproducible level of apoB RNA editing (3% ± 0.2% S.D., n = 3) (Fig. 7). A higher efficiency of editing on the transiently expressed apoB RNA occurred in the APOBEC-1 expressing NGP cell line (5% ± 0.3% S.D., n = 3). Similar results have been obtained with the A172 cell line and its APOBEC-1-expressing counterpart (data not shown). Moreover, identical results were obtained when apoB RNA was expressed through stable transfection in these cells (data not shown). The data clearly indicate that an APOBEC-1-stimulated editing response is possible in neural tumor cells and therefore auxiliary factors involved in apoB RNA editing must be present. Comparatively, however, neuronal cell editing efficiency is approximately one third of that seen on the endogenous apoB transcript in wild-type McArdle cells and suggests that auxiliary factors and APOBEC-1 may be limiting in the neuronal cell lines. Taken together with the data in Figure 6, these findings suggest that although apoB and NF1 base modification RNA editing mechanisms may involve similar *cis*-acting elements, they may not involve the same catalytic subunit or auxiliary factors.

DISCUSSION

Genetics of neurofibromatosis

von Recklinghausen neurofibromatosis or neurofibromatosis type I (NF1) is a hereditary disease predisposing to neoplasia which affects approximately 1 in 3500 live births in the United States, with about half of all cases occurring with no prior family history (45,46). In view of the relatively high rate of sporadic occurrence of NF1, it is expected that many different mutations in the NF1 gene would be detectable. Despite this expectation, relatively few mutations have been detected and no common sites for mutation have been identified.

Patients affected by NF1 may have any combination of clinical manifestations, including café au lait spots, Lisch nodules of the iris, learning disabilities, skeletal abnormalities, and benign or malignant tumors of the central and peripheral nervous systems (47). Interestingly, though the disease is autosomal dominant and fully penetrant, phenotypic expression displays a high degree of variability, even between members of affected families (45). The broad range of manifestations observed and the phenotypic variability between siblings inheriting the same disease allele are difficult to explain if mutations in the NF1 gene alone are responsible. Recently Easton and co-workers (48) presented statistical evidence suggesting the involvement of a locus, distinct from the NF1 locus, that affects the clinical phenotype in NF1 patients. This hypothesis is very intriguing in light of data presented here demonstrating the occurrence of editing factors in neural tumor cell lines and editing of human NF1 mRNA.

The functional homology between NF1 and the mammalian and yeast GAPs (40–42) suggests that the NF1 gene plays a role in a signal transduction pathway involved in regulating cell proliferation. These homologies also suggest that the NF1 gene product is a tumor suppressor and that abnormal cell growth may occur when the NF1 gene product is absent or inactive. To date, biallelic inactivation of the NF1 gene through mutations is considered to be the cause of NF1 functional inactivation.

A '2-hit' mechanism, indicative of a tumor suppressor gene (49), has been demonstrated previously in malignant NF1 tumors (50). It is interesting to note that aberrantly high editing activity could be equivalent to functionally inactivating both NF1 alleles. We detected editing in all cells studied although the proportion of NF1 mRNA that underwent editing varied among different tumor cell types and was markedly higher in solid tumor tissues.

Potential functional consequences of NF1 mRNA editing

The data presented here raise the possibility that RNA editing may be an additional means by which human neurofibromin could be functionally inactivated. Protein translated from edited NF1 mRNA would be truncated N-terminal to the GRD and would therefore presumably be incapable of GTPase activity and of ras inactivation. The functional consequence of 17% NF1

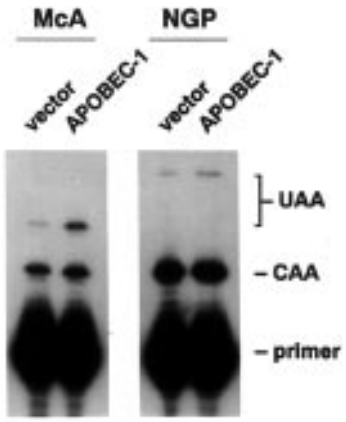

Figure 7. Enhanced apoB RNA editing efficiency in McArdle and NGP cells by APOBEC-1. Stable transfectants of McArdle (McA) and NGP cells were created with either pRc/CMV vector alone (vector) or APOBEC-1 cDNA. Editing activity on the endogenous apoB gene transcripts from both types of McArdle cells was assayed and quantified as described in Materials and Methods. For both types of NGP cells human apoB RNA was transiently expressed and assayed after 48 h for base-modification editing. Bands corresponding to unextended primer (primer), primer-extension products from unedited apoB RNA (CAA) and primer-extension products from edited apoB RNA (UAA) are indicated to the right. ApoB RNA editing efficiency in McArdle and NGP vector transfected cell background was 16% ± 1.5% S.D. and 3.0% ± 0.2% S.D. (n = 3) respectively and for McArdle and NGP APOBEC-1 transfected cell background was 29% ± 1.5% S.D. and 4.6% ± 0.3% (n = 3) respectively.

mRNA editing on neurofibromin function in tumor tissues remains to be evaluated. In this regard, our assays of NF1 RNA editing were performed on RNA isolated from whole tumor tissues which are heterogeneous in both cell type and stage of progression through the cell cycle. Higher levels of NF1 mRNA editing within a select tumor cell subpopulation remains as a distinct possibility.

The introduction of a premature stop codon has been shown to activate exon skipping (51) and is known to occur in Marfan syndrome (52). Editing of human NF1 within exon 23-1 could therefore potentially have an effect on the utilization of the alternatively spliced exon 23a in human NF1 RNA. Introduction of a premature stop codon could also reduce the NF1 mRNA stability (53). The destabilizing effect of an introduced nonsense mutation has been shown to be greater when it occurs close to the 5′-end of the mRNA (54–56) as would be the case for the stop codon introduced into NF1 mRNA by editing (position 2914 of the 13 kb NF1 transcript). A downstream 'instability element' is required that consists of a pair of in-frame initiation codons and a region complementary to the 18S ribosomal RNA sequence (48,57). These features exist in an edited NF1 mRNA and may consequently contribute to decreased neurofibromin levels.

It is interesting to note that while rat NF1 RNA does not appear to contain an editing site, another mechanism exists which introduces an in-frame stop codon within the GAP-related domain. Exon 23b is alternatively spliced to be included in an NF1 transcript termed type III (58). Introduction of this 41 bp exon results in a frame shift which leads to a downstream nonsense codon. In this regard, editing of NF1 RNA was not evolutionarily conserved but the ability to introduce an in frame stop codon within RNA encoding the N-terminus of the GAP-related domain was conserved.

Patient to patient differences in tissue-specific expression of editing activity could account for the individual variation in disease expressivity. In this regard, tissue-specific, developmental stage-specific, metabolic and age-dependent regulation of editing efficiency has been extensively documented for apoB mRNA (2,3,32,44,59,60). Modulation of the abundance or activity of rate-limiting, *trans*-acting factors of the NF1 editosome could dramatically affect the extent to which functional inactivation of NF1 through RNA editing exerts a dominant negative effect. Variations in the level of NF1 RNA editing may also contribute to the high number of spontaneous NF1 cases.

Mechanism of NF1 mRNA editing

Our data suggest that NF1 mRNA editing should be classified in the base-modification category of mRNA editing. Identification of NF1 mRNA as an editing substrate and localization of editing to C2914 through a mooring sequence homology search suggest that the *cis*-acting elements involved in NF1 editing are similar to those involved in apoB mRNA editing (reviewed in Smith, ref. 61). Editing of apoB and NF1 mRNA in transfected cells and by cell extracts *in vitro* suggests that all the factors essential for editing of both RNAs exist in rat hepatoma and human neural tumor cell lines.

We were able to specifically address the involvement of the catalytic subunit of the apoB editing complex, APOBEC-1, in NF1 RNA editing by introducing a recombinant expression vector carrying that cDNA into A172 and NGP cells. In both instances the level of NF1 editing observed was refractory to over-expression of the recombinant cytidine deaminase relative to cells transfected with the vector alone. The neuronal cell lines had the capacity, though limited, to edit apoB RNA in an APOBEC-1-dependent manner and therefore auxiliary factors required for APOBEC-1 function are expressed in these cells. Nonetheless, we cannot rule out the possibility that NF1 RNA editing might also require auxiliary factors distinct from those involved in apoB RNA editing. Though unexpected, this intriguing result raises the possibility of gene-specific factors in RNA editing. In this regard, cells might be able to differentially regulate editing on select RNAs sharing common *cis*-acting editing motifs if the cognate editosomes did not share catalytic subunits. RNA editing site specificity may therefore arise through the interaction of the catalytic subunit with auxiliary factors in the context of the editosome.

In conclusion, the ability to predict RNA editing in NF1 using information available for the behavior of wild-type and mutant editing motifs in apoB RNA presents a new opportunity to evaluate how general this form of editing might be in regulating the structure and function of proteins. Taken together with WT1 mRNA editing, the finding that NF1 mRNA is edited suggests that mRNA editing may a be a general mechanism for tumor suppressor gene inactivation. Future studies will focus on identifying additional mooring sequence-dependent RNA editing substrates from which further insight might be gained into the complexity of *trans*-acting factors involved and the regulation of their activity in issues regarding health and disease.

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