ABSTRACT

Mutations at splice sites or surrounding sequences have been reported to cause aberrant splicing. However, splicing errors can also occur without sequence alterations. We investigated three tumor suppressor genes for aberrant splicing in tumors. At a low frequency per exon it was found in five of seven of the investigated in-frame exons of the neurofibromatosis type 1 (NF1) gene, in two of three exons of the neurofibromatosis type 2 (NF2) gene, and in one of three exons of the tuberous sclerosis 2 gene. It was detectable in all of the human tumor tissues tested (NF1 neurofibroma, sporadic intramedullar neurinoma, sporadic meningiomas, NF2 schwannoma, NF2 meningioma, basalioma, and naevus) as well as in cultured tumor cell lines and cultured primary cells. Hence, our data show that aberrant splicing is a very common process. According to simulations of the secondary structures of the pre-mRNA, we suggest that aberrant splicing is attributable to the rare occurrence of alternative structures at the splice donor site, which are not recognized by the splice machinery. In HeLa cells, aberrant splicing is found to be increased at elevated temperatures and low pH in vitro, conditions often found in tumor tissues. In three tumor tissues tested for one NF1 exon, we found approximately twice the amount of aberrant transcript as in normal tissues. Therefore, we suggest that the increase in aberrant splicing caused by environmental factors represents an additional mechanism for the reduction of the amount of tumor suppressor mRNA in the absence of relevant mutations in the tumor.

INTRODUCTION

Mutations in gene sequences important for the splicing machinery can result in exon skipping and, via instability of the altered transcript or the shortened protein, in functional loss of the allele, as shown for the tumor suppressor gene NF1 (1, 2). Exon skipping, as shown for several genes (3–9), occurs also without mutations at a low frequency in vitro. NF1 is one of the most common autosomal dominant cancer diseases in humans, characterized by multiple tumors of the peripheral nervous system, brain tumors, and malignant tumors. The gene responsible for NF1 on chromosome 17 encodes a mRNA containing up to 60 exons (10). The NF2 gene, located on chromosome 22, is organized in 16 exons. The main symptoms of NF2 are the bilateral nervous system, brain tumors, and malignant tumors. The gene responsible for NF1 on chromosome 17 encodes a mRNA containing up to 60 exons (10). The NF2 gene, located on chromosome 22, is organized in 16 exons. The main symptoms of NF2 are the bilateral schwannomas of the nerve acusticus (11). The TSC2 gene, located on chromosome 16, comprises 41 exons. Complete inactivation of TSC2 leads to formation of hamartomas (12). We report on aberrant splicing of these three tumor suppressor genes in vivo. The presence/absence of aberrant splicing of several in-frame exons was investigated in human dermal and brain tumors, tumor cell cultures, and primary cells. Not all of the exons analyzed were aberrantly spliced. Simulations of the secondary structures of the pre-mRNAs showed that higher free energy structures are altered around exons, which are aberrantly skipped. As shown, splicing is influenced in vitro by temperature, cellular stress (8), or pH of the medium (13). Aberrant splicing might also be influenced by the above environmental factors, which are often altered in tumor tissues. Therefore, we investigated tumor and normal tissues for aberrant splicing. Although aberrant splicing occurs in normal cells at a low level, we suggest that increased aberrant splicing because of environmental factors, in the absence of a corresponding mutation, could represent an additional mechanism for tumor progression by reducing the amount of wt tumor suppressor mRNA.

MATERIALS AND METHODS

Tissues, Cell Culture, and RNA Isolation. Tissue from human basalioma, naevus and NF1 neurofibroma (a gift from K. Kunzi-Rapp, University of Ulm, Ulm, Germany), sporadic intramedullar neurinoma, sporadic meningiomas, NF2 schwannoma, NF1 and NF2 meningioma, astrocytoma grade IV, spongioblastoma, and skin and prostate biopsies from healthy donors were stored directly after excision in liquid nitrogen or in RNAlater (Ambion, Austin, TX). The histology of the tumors was analyzed and recorded in parallel. Cells from a neurofibroma of NF172 (NF172T), melanoma cells (BM), glioblastoma multiforme cells (MG), HeLa cells, a human hematoma cell line (HuH7), primary fibroblasts from dermis (FC7, FC11, and FC14), or from nervous suralis (N26) of healthy donors were cultured with DMEM containing 10% fetal bovine serum. Primary melanocytes (MC13) and keratinocytes (KC6) of healthy donors were cultured as described (14). Cells were detached and lysed for RNA extraction. Peripheral blood cells were isolated from fresh blood samples of two healthy donors (C14 and C15). Subconfluent cultures of HeLa cells or FC14 were incubated for 1 day at temperatures ranging from 7°C to 41.5°C or in a medium with a pH of 6.3–7.4, lysed in the culture flasks on ice and harvested with a cell scraper for RNA extraction. Total RNA was isolated using the RNeasy spin columns (Qiagen, Valencia, CA), quantified by measuring the absorbance at 260 nm, its quality verified on a 0.8% agarose gel. The histology of the tumors was analyzed and recorded in parallel. Cells from a neurofibroma of NF172 (NF172T), melanoma cells (BM), glioblastoma multiforme cells (MG), HeLa cells, a human hematoma cell line (HuH7), primary fibroblasts from dermis (FC7, FC11, and FC14), or from nervous suralis (N26) of healthy donors were cultured with DMEM containing 10% fetal bovine serum. Primary melanocytes (MC13) and keratinocytes (KC6) of healthy donors were cultured as described (14). Cells were detached and lysed for RNA extraction. Peripheral blood cells were isolated from fresh blood samples of two healthy donors (C14 and C15). Subconfluent cultures of HeLa cells or FC14 were incubated for 1 day at temperatures ranging from 7°C to 41.5°C or in a medium with a pH of 6.3–7.4, lysed in the culture flasks on ice and harvested with a cell scraper for RNA extraction. Total RNA was isolated using the RNeasy spin columns (Qiagen, Valencia, CA), quantified by measuring the absorbance at 260 nm, its quality verified on a 0.8% agarose gel. DNase I treated (Life Technologies, Inc., Rockville, MD), and stored at −70°C. Total RNA (1 μg) was reverse transcribed using random hexamers (SuperScript; Life Technologies, Inc.). The cDNA derived from several tissues of human adults was purchased from OriGene (Rockville, MD).

Detection of NF1 Δ-E8. PCRs were carried out with 3 μl of cDNA and the primers NF1/NF335 (Table 1), wt product: 326 bp, Δ-E8: 203 bp. To avoid cross-contamination during the electrophoresis in bufferless, precast 2% agarose gels (E-gels; Invitrogen, Carlsbad, CA), the PCR products were loaded one empty lane apart. The gel fragment, where the PCR product of 203 bp should reside, was excised with a fresh scalpel, washed three times in H₂O, and the DNA extracted using the QIAquick Gel Extraction kit (Qiagen) and reamplified. To exclude processed pseudogenes as a source of shortened amplification products, genomic DNA from the cells was amplified with the primers NF1/NF335. To rule out PCR artifacts, a plasmid containing only the NF1 wt sequence from exon 4a to exon 11 was constructed with the TOPO TA cloning system (Invitrogen) and a RT-PCR product made with primer pair

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2 To whom requests for reprints should be addressed, at Universitätsklinikum Ulm, Abteilung Humangenetik, D-89070 Ulm, Germany.
was 3.27
in these measurements, three cDNAs were made from mixing NF172T RNA, (26, 28, or 30 cycles) the band intensities of the reamplification products were (GFX kit; Amersham Pharmacia Biotech, Piscataway, NJ) and reamplification various temperatures or pH, three independent PCRs each were performed for
and measured on PhosphorImager. Lastly, for measurement of the frequency at
cycles for PCR amplification and reamplification, respectively.

Investigations of Additional NF1 Exons, NF2, and TSC2. The primer pairs used for detection of aberrant splicing in NF1, NF2, and TSC2 and the respective PCR product length are shown in Table 1. The PCR products were either sequenced on an ABI 373 sequencer (PE Applied Biosystems, Foster City, CA) or digested by restriction endonucleases to confirm the presence of
pairs used for detection of aberrant splicing in
and the
product in normal cells, PCR products were made from HeLa, C14, and serial
expression was measured with TAMRA-labeled RT-PCR products made with
for measurement of the
NF1
/Nf335 and separated on an ABI 310 sequencer (data not

Frequency of NF1 Exon 10b Skipping. Transcripts lacking NF1 exon 10b were amplified with skipt-specific primer NF10a/c-H (5'-CAATACGAAGT-GCACCAGATTCT-3', pos. 1376–1392 + 1528–1532, in italics: nucleotide change (C→T) conveying the specificity) and TAMRA-labeled primer Nf732, change (C
Cells.

Simulation of Minimum Free Energy Structures of NF1 pre-mRNA. For the calculation of minimum free energy structures, mfold version 3.0 (15) was used. The kinetic of the folding process is determined by the transcription rate, the formation of stable and metastable structures (16), and the time window within which the naked RNA can fold before heterogeneous nuclear ribonucleoproteins associate with it (17). We based our calculation on the knowledge of the functional sequences (e.g., splice sites) and the assumed time window. To mimic transcription, the sequence was built up by small sections. Section length and sequence starting points were varied until characteristic window. To mimic transcription, the sequence was built up by small sections.

RESULTS

Low Frequency of NF1 Δ-E8 in HeLa and Peripheral Blood Cells. We have found a germ-line mutation G to A at position 1185 + 1(G→A) of the NF1 gene in peripheral blood cells of the NF1 patient NF172 leading to Δ-E8 with equal expression of both alleles. In cDNA from HeLa cells and blood cells of healthy donors (C14 and C15) this Δ-E8 transcript was not observed after amplification. How-
ever, if the region of the agarose gel corresponding to Δ-E8 was reamplified, the Δ-E8 transcript was detected (Fig. 1A). By sequencing we confirmed that the shortened transcripts indeed lacked exon 8 (Fig. 2). Sequencing of the branch point, 5′ and 3′ splice sites of the NF1 exons 7 to 9, showed no mutations. Amplification of genomic DNA of HeLa and C14 cells with the same primers yielded no product, excluding the amplification of a processed pseudogene. Amplification, gel extraction, and reamplification of plasmid DNA, containing only the wt NF1 sequence of exon 4a-11. PCR products separated on 1.5% agarose gels stained with ethidium bromide. Lane 1, plasmid, amplification; Lane 2, blood cells of a healthy donor (C14), amplification; Lane 3, blood cells of C14, reamplification; M, 100-bp ladder; wt, 326-bp; Δ-E8, 203-bp.

ABERRANT SPlicing IN Tumors

Simulating NF1 pre-mRNA Secondary Structures. It is well known that folding of pre-mRNA molecules is dependent on the sequence and the given conditions in vitro or in vivo. We suggest that...
the secondary structures obtained with the algorithms described (17, 19) are useful approximations for the functional structures in vivo (20). It was possible to interpret observed alternative splicing caused by mutations in the NF1 and hypoxanthine-guanine phosphoribosyltransferase gene (21, 22) on the basis of predicted alterations of the minimal free energy secondary structures. Aside from the lowest free energy secondary structures, most probably formed under physiological conditions, alternative structures with higher free energies are possible. Their formation may to a certain extent be dependent on conditions such as temperature and pH. We suggest that these alternative structures, if not recognized by the splice machinery, could be a cause of aberrant splicing. Mutations leading to exon skipping in the NF1 gene are predominantly located at the splice donor sites (1, 2).

Therefore, we have concentrated our investigations on these regions. The mutation in NF1 intron 8 [1185 + 1(G→A)] and a second mutation at this site [1185 + 1(G→U); Ref. 23] cause exon 8 skipping. The minimal free energy secondary structure of the intron 8 splice donor site of both mutated NF1 pre-mRNAs was simulated and compared with the wt structure (Fig. 5, A–C). Remarkable changes were found in the structure motifs of the splice donor sequence region in mutated pre-mRNA. We suggest the altered structures in the splice donor region to be the cause of exon skipping.

The simulation of minimum free energy secondary structures of pre-mRNA may detect these alterations. We suspect that the aberrant splicing in 5 of 7 of the NF1 exons correlates with differences in the probability of the primary transcripts to achieve the regular structure necessary for normal splicing. To investigate this hypothesis, secondary structures of NF1 pre-mRNA splice donor sequences of exons undergoing aberrant splicing (NF1 exon 8 and exon 10b) and those that do not (NF1 exon 10a and exon 10c) were simulated. Structures with higher free energies are presumably rarely formed, and their occurrence is dependent on the temperature. Their influence on the splice process was not investigated up to now. For exon 8 and exon 10b, which are skipped, structures that immediately follow the minimal free energy structure in the energy scale show altered structure motifs in the splice donor site regions (exon 8; Fig. 5, A and D). For exon 10a and exon 10c where exon skipping was not detectable, such structure alterations were not predicted (exon 10c; Fig. 6, A and B). The splice site score values calculated as described (24) do not correlate with the presence/absence of aberrant splicing.

Table 2: aberrant splicing of NF1 exons in vivo and in vitro

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<tr>
<th>NF1</th>
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<th>Δ-E8</th>
<th>Δ-E9</th>
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Fig. 4. aberrant splicing of NF1 exon 10b in human tumors. 1, NF1 neurofibroma; 2, sporadic intramedullar neurinoma; 3, keratinocytes; 4, keratinocytes H2O; 5, peripheral nerve cells; 6, melanocytes; 7, melanocytes H2O; 8, basaloma; 9, HuH7 cells; 10, 100-bp ladder. wt, 326 bp; Δ-E8, 203 bp; Δ-E9/10a (1213–1299), 239 bp. The 239 bp reamplification product detectable in several tumors represents another aberrant splice product lacking parts of NF1 exon 9 and 10 (Δ-E9/10 (1213–1299)) as shown by sequencing.

Fig. 3. aberrant splicing of NF1 exon 8 skipping in human tumors (A), primary cell cultures, and cell lines (B). Reamplification PCR products separated on 2% agarose gels stained with ethidium bromide. A1, NF1 neurofibroma; 2, sporadic intramedullar neurinoma; 3 and 4, sporadic meningiomas; 5, NF2 vestibular schwannoma; 6, NF2 meningioma; 7, dysplastic naevus; 8, basaloma; 9, HuH7 cells; 10, H2O; M, 100-bp ladder. B1, peripheral nerve cells; 2, melanocytes; 3, keratinocytes; 4, fibroblasts; 5, H2O; 6, C14; 7, C15; 8, glioblastoma cells; 9, BM cells; 10, H2O; M, 100-bp ladder. wt, 326 bp; Δ-E8, 203 bp; Δ-E9/10a (1213–1299), 239 bp. The 239 bp reamplification product detectable in several tumors represents another aberrant splice product lacking parts of NF1 exon 9 and 10 (Δ-E9/10 (1213–1299)) as shown by sequencing.

Fig. 5. Simulated secondary structures for NF1 intron 8 splice donor site. A. wt; B, mutation 1185 + 1(G→U); C, mutation 1185 + 1(G→A); A–C, minimal free energy; D, wt higher free energy.
Occurrence of Aberrant NF1 Exon 8 Splicing Is Temperature- and pH-dependent. The amount of NF1 exon 8 skipping was measured by the use of gel extraction and reamplification (fourth method for frequency measurement) in HeLa cells and primary fibroblasts (FC7), which were cultured at different temperatures for 2 days. In both cases, aberrant splicing clearly increased at higher temperatures as well as low temperatures, especially 7°C (Fig. 7A). Radioactive PCR of HeLa cDNAs confirmed this. The method of gel extraction and reamplification was tested beforehand for possible PCR artifacts. cDNAs were made from different mixtures of NF172T and HeLa RNA. They contained 2.5% (1:12), 1.5% (1:25), and 0.8% (1:49) exon 8 skipped transcript as shown by measurement on an ABI 310 sequencer. The separated PCR products of these cDNAs were then subjected to gel extraction and reamplification. As shown in Fig. 8 the amount of the measured reamplification products corresponds to the expected amount. Although there is variation there is no evidence for PCR artifacts obscuring the measurement. In addition to temperature, cellular stress was also investigated. The amount of Δ-E8 product increased in HeLa cells cultured at low pH (Fig. 7B). Furthermore, as shown by radioactive PCR, reduction of fetal bovine serum from 10% to 1% increased the amount of NF1 Δ-E8 in cultured fibroblasts derived from peripheral nerve (N26, 370%) or dermis (FC11, 160%).

The Level of Aberrant Splicing Is Elevated in Tumor Tissues. The level of NF1 exon 10b skipping was investigated with skip-specific PCR in three tumor tissues (NF1 meningioma, sporadic spongioblastoma, and astrocytoma grade IV) in comparison to peripheral blood cells of a healthy donor (set to 100%), skin (124%), and two prostate biopsies (56% and 69%) of healthy donors. The amount of exon 10b skipped transcript was elevated in all three of the tumor tissues (197% in meningioma, 227% in spongioblastoma, and 232% in astrocytoma).

Aberrant Splicing in Exons of NF2 and TSC2 in Tumor Tissues. Aberrant splicing also occurs in other tumor suppressor genes such as NF2 and TSC2. Using primers flanking NF2 exon 9 to exon 11, shortened PCR products were found after reamplification of the cDNAs from tumors and cultured cells (Fig. 9A). Sequencing confirmed the presence of NF2 Δ-E10, which was detectable in all of the tumors and cultured cells. NF2 Δ-E9/10 could be seen in NF1 neurofibroma, sporadic intermedullar neurinoma, NF2 meningioma, and

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**Fig. 6.** Simulated secondary structure for NF1 intron 10c splice donor site. A, wt minimal free energy; B, wt higher free energy.

**Fig. 7.** Amount of NF1 Δ-E8 in HeLa and fibroblasts (FC7) cultured at various (A) temperatures (7–41.5°C) and (B) pH (6.3–7.3); bars, ± SE.

**Fig. 8.** Exclusion of PCR artifacts. cDNAs with defined amount of NF1 Δ-E8 were subjected to three independent amplification, gel extraction, and reamplification reactions. The amounts of reamplification products were normalized on the 2.5% of cDNA. The resulting mean percentage of three measurements were plotted against the expected percentage of the ABI measurement; bars, ± SE.

**Fig. 9.** Reamplification PCR products of NF2 exons 9–12 (A) and TSC2 exons 14–17 (B) in human tumors and cultured cells. 1, HeLa; 2, MC13; 3, FC11; 4, C14; 5, C15; 6, NF1 neurofibroma; 7, sporadic intramedullar neurinoma; 8 and 9, sporadic meningiomas; 10, NF2 vestibularis schwannoma; 11, NF2 meningioma; 12, dysplastic naevus; 13, basalioma. A: M, 100-bp ladder; NF2 wt, 591 bp; NF2 Δ-E10, 477 bp; NF2 Δ-E9/10, 402 bp. B: M, 20-bp ladder; TSC2 wt, 627 bp; TSC2 Δ-E15, 510 bp.
basalioma. Additional PCR products visible in Fig. 9A consisted of at least two overlapped sequences as shown by sequencing. Using primers flanking TSC2 exon 14 to exon 17 aberrant products were found after reamplification in addition to the wt product. By sequencing the 510-bp product we confirmed skipping of TSC2 exon 15. This transcript was detectable in all of the tumors, tissues, and cell cultures tested except in a sporadic intramedullar neurinoma and meningioma (Fig. 9B). Amplification of gDNA with the NF2 or TSC2 primers did not result in products of the size described for reamplification of cDNA. In C14 and a sporadic meningioma, radioactive PCR showed a ratio of <0.5% aberrant splice products in NF2 Δ-E10 and TSC2 Δ-E15. Simulation of the secondary structures of flanking TSC2 least two overlapped sequences as shown by sequencing. Using primers 510-bp product we confirmed skipping of after reamplification in addition to the wt product. By sequencing the B (Fig. 9). Amplification of gDNA with the tested except in a sporadic intramedullar neurinoma and meningioma.

DISCUSSION

The Physiological Aberrant Splicing Occurs Also in Tumors. Recently, aberrant splicing was detected in the NF1 gene in the absence of a germ-line mutation during mutation analysis at the cDNA level in blood cells (25). It seems to be induced by unphysiological culture conditions such as storage of cells at room temperature before RNA extraction (26–28). We also observed aberrant splicing in the NF1, the NF2 and the TSC2 genes in cells maintained at standard culture conditions, and, more importantly, in vivo in tumor tissues. The present investigation along with data from other groups suggests that aberrant splicing of a single exon is a general and physiological phenomenon. In our sample survey we detected it for ~60% of the tested in-frame exons. It remains to be shown how representative this finding is. Out-of-frame exon-skipped transcripts are usually recognized and disintegrated by the nonsense-mediated mRNA decay (18) and, therefore, are not detected. There seems to be no such degradation pathway for in-frame exon-skipped transcripts. It has been demonstrated that in NF1 cold stress induces insertion of a cryptic exon (26) plus multi exon splice errors (27). Our data show increased aberrant splicing at both low and high temperatures. One reason for this increase may be the reduced expression or inactivation of one or more splice factors, resulting in a reduced accuracy of the splice machinery. Another conceivable explanation is a structural alteration of the pre-mRNA leading to a reduced recognition by the splice machinery. Specific pre-mRNA structure motifs play an important role in the regulation of the splice process. Therefore, we compared the minimal free energy structures of splice donor sites with possible alternative structures with higher free energies. The amount of these alternative structures may be increased under unphysiological conditions such as increased temperature or acidity. We suggest that this mechanism is responsible for aberrant splicing.

In Tumor Suppressor Genes with a Large Number of Exons, Increased Aberrant Splicing May Represent an Additional Mechanism of Reduction of the wt mRNA Amount. Although our investigations show that the amount of aberrant splice products is low for a single exon, it may be of biological importance in genes with many exons. The increase in aberrant splicing through environmental factors may play an especially important role in tumor suppressor genes where the exact gene dose is crucial. In hereditary cancer syndromes caused by germ-line mutations in tumor suppressor genes, tumors are attributable to the loss of the wt allele leading to additional reduction of the gene product. Several mechanisms may be involved in this reduction, including presence of a mutation in the second allele of the tumor suppressor gene, mRNA editing as shown in NF1 (29), proteolysis as exemplified by the calpain-dependent proteolysis of the NF2 protein schwannomin (30), or alternative splicing as shown for a brain-specific exon in Bin1 eliminating the activity of the tumor suppressor in melanoma (31). Increased aberrant splicing may represent an additional mechanism for the reduction of the amount of wt tumor suppressor mRNA without mutation. The following calculation example concerning NF1 may clarify this. Assuming an inaccuracy of 1% aberrant splicing per exon in 40 of the 60 NF1 exons only 66.8% [(1–0.01)^40] functional mRNAs would be produced normally by transcription. An increase of the aberrant splicing by environmental factors to 2% per exon would result in 44.5% accurate NF1 mRNA in normal cells or 22.3% in NF1 cells. It remains to be investigated in detail whether aberrant splicing occurs in vivo in this range, for instance in NF1 tumors. Additionally, in regard to sporadic tumors, increased aberrant splicing may represent an additional mechanism to reduce the amount of functional tumor suppressor mRNA. Our results show increased aberrant splicing at high temperature and low pH. These are conditions often found in tumor tissues. Investigation of one exon skip in three tumor tissues indeed showed approximately twice the amount of exon-skipped transcript as in the control. Aberrant splicing may also limit the maximal number of in-frame or total exons of a gene in evolution. In humans, the majority of genes comprise <13 exons, the minority >30, whereas only a very few genes contain >100 exons (32). In the case of a human gene with 100 exons, aberrant splicing of 1% per exon results in only 36.6% [(1–0.01)^100] accurate mRNAs. This idea is additionally supported by the fact that genes that are dependent on high transcriptional fidelity, such as the G protein-coupled receptors, are predominantly intronless (33).

ACKNOWLEDGMENTS

We thank E. Winkler for expert technical assistance. We also thank R. Mueller and H. Lattke for the discussion.

REFERENCES

ABERRANT SPLICING IN TUMORS


Aberrant Splicing in Several Human Tumors in the Tumor Suppressor Genes Neurofibromatosis Type 1, Neurofibromatosis Type 2, and Tuberous Sclerosis 2

Dieter Kaufmann, Werner Leistner, Petra Kruse, et al.