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Deregulated expression of *PAX5* in medulloblastoma

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ABSTRACT **Medulloblastoma is a pediatric brain tumor originating in the human cerebellum. A collection of 23 medulloblastomas was analyzed for expression of the developmental control genes of the *PAX* and *EN* gene families by RNase protection and *in situ* hybridization. Of all nine *PAX* genes investigated, only *PAX5* and *PAX6* were consistently expressed in most medulloblastomas (70 and 78% of all cases, respectively), as were the genes *EN1* (57%) and *EN2* (78%). *EN1*, *EN2*, and *PAX6* genes were also expressed in normal cerebellar tissue, and their expression in medulloblastoma is consistent with the hypothesis that this tumor originates in the external granular layer of the developing cerebellum. *PAX5* transcripts were, however, not detected in the neonatal cerebellum, indicating that this gene is deregulated in medulloblastoma. In the desmoplastic variant of medulloblastoma, *PAX5* expression was restricted to the reticulin-producing proliferating tumor areas containing undifferentiated cells; *PAX5* was not expressed in the reticulin-free nonproliferating islands undergoing neuronal differentiation. These data suggest that deregulated expression of *PAX5* correlates positively with cell proliferation and inversely with neuronal differentiation in desmoplastic medulloblastoma.**

Medulloblastoma is a highly malignant tumor of the cerebellum (1) and represents the most common brain neoplasia of childhood, for which long-term prognosis remains dismal despite advances in therapeutical treatment (2). Medulloblastoma is thought to arise by neoplastic transformation of undifferentiated precursor cells present in the external granular layer of the embryonic cerebellum (3). These precursor cells normally migrate inward during the first 2 years of life to give rise to the differentiated granule neurons of the internal granular layer (4, 5). In agreement with the postulated origin in the external granular layer (3), medulloblastomas often display a tendency toward neuronal differentiation *in vivo* (6, 7).

To date little is known about the molecular mechanisms underlying the pathogenesis of medulloblastoma. None of the common genetic alterations identified in other neuroectodermal tumors such as oncogene amplification (8) or point mutation of the *p53* (9) and N-*ras* (10) genes have so far been uncovered as key events in the formation of medulloblastoma. The identification of regulatory genes that are expressed in this pediatric brain tumor may provide an alternative approach to gain insight into the molecular aspects of tumor formation. Members of the *pa*ired bo*x*-containing (*PAX*) gene family are potential candidate genes, as they code for transcription factors that are expressed in specific regions of the brain and contribute to patterning of particular brain structures. These genes have been implicated in the control of cell fate specification, proliferation, and/or migration of neuroectodermal precursor cells during development (for review, see refs. 11–13). For example, targeted inactivation of the *PAX5* gene leads to hypoplasia of the inferior colliculus in the posterior midbrain, suggesting that this control gene is essential for the proliferation of a population of localized neuroectodermal precursor cells (14). Furthermore, overexpression of *PAX* genes appears to elicit transformation of rat fibroblasts *in vitro*, thus identifying these genes as potential oncogenes (15). In addition, the *PAX3* and *PAX7* genes are activated in alveolar rhabdomyosarcoma, a myogenic tumor of childhood, by specific chromosomal translocations that consistently involve one of the two *PAX* loci (16, 17).

For these reasons, we set out to analyze the expression of *PAX* family members in a large collection of medulloblastomas. In addition, we have investigated expression of the homeobox-containing genes *EN1* and *EN2*, which are transcribed in the embryonic brain region giving rise to the cerebellum (18). *PAX5*, *PAX6*, *EN1*, and *EN2* genes were expressed at high frequency in the different medulloblastomas. In contrast to PAX6, EN1, and EN2 mRNA, transcripts of the *PAX5* gene could not be detected in normal cerebellar tissue of neonates and adults, suggesting that *PAX5* expression is deregulated during the genesis of medulloblastoma.

MATERIAL AND METHODS

Histological and Immunohistochemical Analyses. Aliquots of each tumor were snap-frozen for RNA extraction and cryostat sectioning. The remaining tumor material was fixed for 3 hr at room temperature in PBS containing 4% (vol/vol) formaldehyde and then processed for paraffin sectioning. Sections of 3 μm thickness were either stained with hematoxylin/eosin or used for immunostaining with a polyclonal rabbit anti-bovine glial fibrillary acidic protein antibody (Dako), a rabbit anti-human neuron-specific enolase (NSE) antibody (Dako), or a rabbit anti-human synaptophysin antibody (Dako) or with the monoclonal antibody MIB-1 (Dianova, Hamburg, F.R.G.) detecting the Ki-67 antigen (19). Reticulin staining was performed by the silver impregnation method of Gomöri (see ref. 19).

***In Situ* Hybridization Analysis.** Human *PAX5* (20) and *PAX6* (21) cDNAs were cloned into the polylinker of plasmid Bluescript KS. Sense and antisense RNA probes were prepared by *in vitro* transcription of linearized pKS-PAX5 and pKS-PAX6 DNA with T3 or T7 RNA polymerase in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The labeled transcripts were partially hydrolyzed to an average length of 50–100 nt and hybridized to tumor sections followed by detection with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Boehringer Mannheim).

PCR Oligonucleotides. The following oligonucleotides were used: PAX2, GCGGTCGACTTTCAACCCAACGCCGGATGG and GCGAAGCTTTCGCAAGTGCTTCCGCAAACTG; PAX5, GCGAGATCTCATGGAGGAGTGAATCAGCTT and GCGAAGCTTACTGCTGTACTTTTGTCCGGAT; PAX6, GCGGTCGACAGGTGTCCAACGGATGTGTGAG and GCGAAGCTTGGTATGTTATCGTTGGTAC; PAX9, GCGGGATCCACCGTGGTGAAACACATCCGGAC and

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Abbreviations: PNET, primitive neuroectodermal tumor; NSE, neuron-specific enolase; CNS, central nervous system.

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GCGAAGCTTGGCGTCGGCTGGTGCTGCTTGTA; EN1, GCGGGATCCAGACTCAAGGCGGAGTTCCAGGCA and GCGAAGCTTCTACTCGCTCTCGTCTTTGTCCTG; EN2, GCGGGATCCAGGCTCAAGGCCGAGTTCCAGACC and GCGAAGCTTGTACAAGCCCTGTGCCATGAGGTGC; WNT1, GCGGGATCCCTCCGCCGGGGTCACCCATTCGG and GCGAAGCTTGGTTCATGAGGAAGCGCAGGTC.

RNase Protection Analysis. Total RNA (10 μg) prepared from tumor and normal tissues was used for RNase protection analysis as described (20). RNA probes were generated by SP6 transcription of cDNA cloned in the antisense orientation into the polylinker of pSP64. *PAX2*, *-5*, *-6*, and *-9*, *EN1* and *-2*, and *WNT1* cDNA fragments were generated by PCR amplification using the oligonucleotide primers shown above and A-704 cell cDNA (22), hBSAP-1 cDNA (20), or human genomic DNA as template, respectively. The *PAX1* and *PAX3* probes were cloned as a 312-bp *Hin*cII–*Sac* I fragment of clone HuP48 (23) and as a 156-bp *Asp*718–*Hin*cII fragment of clone HuP2 (23), respectively. The *PAX4* and *PAX7* probes were obtained by subcloning a genomic *PAX4* DNA fragment (24) and a 294-bp *Asp*718–*Bst*XI fragment of *PAX7* cDNA (25). The *PAX8* and *GAPDH* probes were as described (22).

RESULTS

Patient Collective. Our collective consisted of 23 medulloblastoma patients. In 14 patients, the medulloblastoma arose in the vermis and thus affected the midline of the cerebellum; in 9 patients, the tumor originated in the lateral cerebellar hemispheres (Table 1). Our study also included 3 patients with PNET originating from the forebrain or the pineal gland, 1 patient with glioblastoma, and 1 patient with anaplastic ependymoma (Table 1). During the course of the study, 3 patients suffered from local recurrence at the original tumor site and underwent additional surgery. Tumor tissues from both surgical procedures were available for 2 of these 3 patients (tumors 973/1128 and 341/912), but for 1 patient (tumor 938) only the recurrent lesion could be analyzed (Table 1). All tumors were identified by histological means as classical medulloblastoma with the exception of one (tumor 341) from a patient who was diagnosed with a desmoplastic medulloblastoma synthesizing abundant reticulin fibers (26). After local recurrence, this tumor had lost its desmoplastic characteristics (tumor 912; Table 1).

Immunohistochemical analysis revealed expression of NSE in 24 medulloblastomas, thus providing evidence for focal neuronal differentiation in these tumors (Table 1). In addition to NSE, synaptophysin was expressed in 11 medulloblastomas, indicating more pronounced maturation along the neuronal lineage in these tumors. Expression of glial fibrillary acidic protein, a marker of astrocyte differentiation, was detectable in neoplastic cells of only 8 medulloblastomas, 7 of which also showed evidence of neuronal differentiation.

Expression of *PAX* and *EN* Genes in Medulloblastoma. To study *PAX* and *EN* gene expression in medulloblastoma, we generated RNA probes for each of the nine human *PAX* genes

Table 1. Patient collective

Bank	Age, years	Sex	Diagnosis	Location	Immunocytochemistry NSE	Syn	GFAP
35	26	F	MB	Hemisphere	+	−	+
89	18	F	MB	Up. vermis	+	−	−
126	18	F	MB	Hemisphere	+	−	−
238	40	M	MB	Hemisphere	−	−	+
248	7	M	MB	Hemisphere	+	−	−
332	5	F	MB	Vermis	+	+	−
341	22	M	Des. MB	Uv. vermis	+	−	+
412	6	M	MB	Uv. vermis	+	−	−
660	8	M	MB	Uv. vermis	+	+	−
662	32	M	MB	Hemisphere	+	−	−
711	34	F	MB	Hemisphere	+	+	−
912	24	M	MB	Vermis	+	−	+
938	12	F	MB	Vermis	+	+	−
961	16	M	MB	Vermis	+	−	+
973	60	M	MB	Uv. vermis	+	+	−
1007	22	M	MB	Vermis	+	+	−
1128	60	M	MB	Vermis	+	+	−
1029	32	F	MB	Vermis	+	−	−
1132	9	M	MB	Uv. vermis	+	+	+
1138	6	M	MB	Hemisphere	+	−	+
1139	31	M	MB	Hemisphere	+	−	−
1200	2	M	MB	Vermis	+	+	−
1211	27	M	MB	Hemisphere	+	−	+
1218	34	F	MB	Up. vermis	+	+	−
1279	1	M	MB	Up. vermis	+	+	−
548	15	M	PNET	Multifocal	+	−	−
557	22	M	PNET	Pineal gl.	+	+	−
1010	5	F	PNET	Fr.-parietal	+	+	−
634	40	M	GB	Fr. cortex	NA	NA	+
1192	0.5	F	An. ED	4. ventricle	NA	NA	NA

Age of each patient at the time of operation is indicated. All tumors arose in the cerebellum except for tumors 548, 1010, and 634 originating in the forebrain and tumor 557 originating in the pineal gland. Recurrent tumors are boxed. Histochemical staining was performed with antibodies against NSE, synaptophysin (Syn), and glial fibrillary acidic protein (GFAP). MB, medulloblastoma; PNET, primitive neuroectodermal tumor; GB, glioblastoma multiforme; ED, ependymoma; Des., desmoplastic; An., anaplastic; Uv., uvula; Up., upper; Fr., frontal; gl., gland; NA, not applicable; F, female; M, male.

(24) and for the homeobox-containing *EN1* and *EN2* genes (18). These probes were used together with a reference *GAPDH* probe for analysis of the tumor panel described in Table 1. RNA isolated from various brain regions of neonatal and adult patients, who died of noncerebral causes, was included in the analysis to control for *PAX* and *EN* gene expression in the normal central nervous system (CNS). Two of these tissues (lobulus quadrangularis and uvula vermis) were derived from the cerebellum, two (inferior and superior colliculus) were from the dorsal midbrain, and one (frontal cortex) was from the forebrain. A representative RNase protection experiment using the *PAX5* probe is shown in Fig. 1, and the results obtained with all *PAX* and *EN* probes are compiled in Fig. 2. In these experiments, expression of four of the *PAX* genes (*PAX4*, *-7*, *-8*, and *-9*) was not detected in any of the tumor samples. Three murine *Pax* genes (*Pax-2*, *-3*, and *-6*) are expressed in different regions of the adult cerebellum of the mouse (13). As shown in Fig. 2, the same genes are also expressed in the adult cerebellum of humans (lobulus quadrangularis and uvula vermis). Of these three genes, only *PAX6* was expressed at significant levels in most medulloblastomas (18 of 23; 78%), while *PAX2* and *PAX3* transcripts were detected only in a subset of tumors. Interestingly, three tumors also contained *PAX1* transcripts, even though expression of the mouse *Pax-1* gene has so far not been found in any region of the CNS (13).

The *PAX5* gene was expressed in most medulloblastomas analyzed, albeit at different levels (Figs. 1 and 2). This finding is particularly striking, since no *Pax-5* transcripts were detected in the neonatal and adult cerebellum of humans (Fig. 2) and mice (13). *PAX5* is, however, expressed in the region of the inferior and superior colliculi (Fig. 2). Normalizing the abundance of the *PAX5* transcripts relative to the control GAPDH mRNA indicated that 16 (70%) out of 23 medulloblastomas expressed the *PAX5* gene at a similar or higher level than did the superior colliculus of neonates (Fig. 1). *PAX5* expression in 9 tumors (40%) was comparable or even higher than that of the clonal B-cell line BJA-B where every cell transcribes the *PAX5* gene (20). *PAX5* expression could not be detected in three control tumors (tumors 548, 634, and 1192) but was observed in two PNETs originating from the pineal gland (tumor 557) and forebrain (tumor 1010).

The *En-2* gene is known to be strongly expressed in the adult cerebellum of the mouse (18). Strong *EN2* expression was also detected in the adult human cerebellum, and lower levels of *EN1* mRNA were found in the same brain region (Fig. 2). Moreover, the *EN1* and *EN2* genes were expressed in 57% (13 of 23) and 78% (18 of 23) of all medulloblastomas, respectively. Thus, these data indicate that the four genes *PAX5*, *PAX6*, *EN1*, and *EN2* are frequently coexpressed in medulloblastoma.

Localized Expression of *PAX5* in Desmoplastic Medulloblastoma. To localize expression of the *PAX5* and *PAX6* genes within the tumor tissue, we next analyzed each medulloblastoma by *in situ* hybridization. Due to differences in RNA quality of the different tumor samples, *in situ* hybridization did not yield interpretable results for all tumors that expressed *PAX5* and *PAX6* based on the RNase protection assays. However, those medulloblastomas that gave rise to *PAX5* and *PAX6* mRNA signals in the *in situ* hybridization analysis indicated that these two genes were homogeneously expressed throughout the tumor. Moreover, expression of both genes was confined to tumor cells and could not be detected in endothelia or surrounding connective tissues (data not shown).

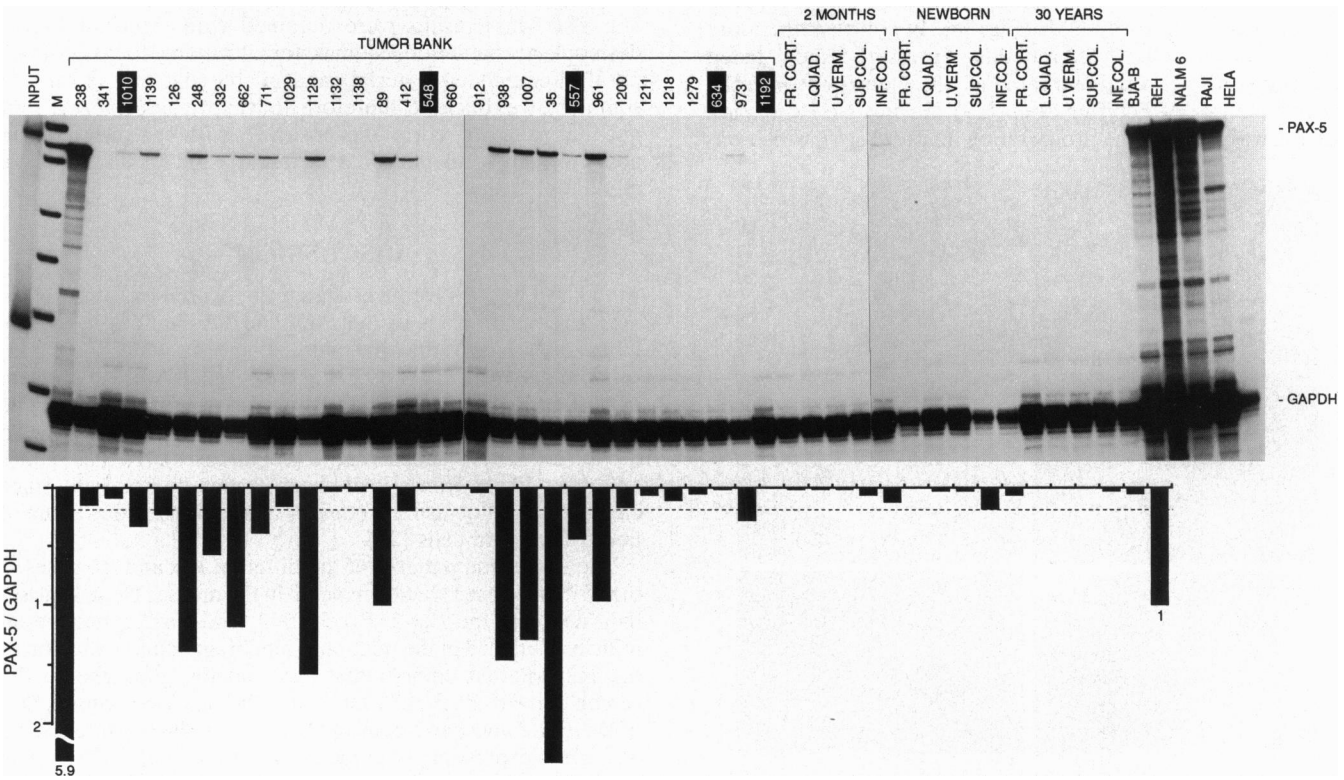

FIG. 1. *PAX5* expression in medulloblastomas. (*Upper*) Total RNA (10 μg) isolated from various medulloblastomas, B-lymphoid cell lines (BJA-B, REH, NALM6, and RAJI), and HeLa cells was analyzed by RNase protection with *PAX5* and *GAPDH* RNA probes. The five tumors diagnosed as PNET, glioblastoma, or ependymoma (see Table 1) are indicated by black overlay. Brain tissues of three patients who died of noncerebral causes were analyzed as control tissues. The sizes of the two RNA probes prior to RNase digestion are shown to the left (lane input). End-labeled pUC19 DNA digested with *Msp* I was used as size marker (lane M). The autoradiograph was exposed for 15 hr, and autoradiographic signals were quantitated on a PhosphorImager (Molecular Dynamics). The *PAX5* transcript level of each tumor and tissue was normalized for *GAPDH* expression and plotted relative to the expression level observed in the BJA-B cell line. (*Lower*) A dashed line indicates the level of *PAX5* expression in the superior colliculus of neonates. Fr. Cort., frontal cortex; L. Quad., lobulus quadrangularis; U. Verm., uvula vermis; Sup. Col., superior colliculus; Inf. Col., inferior colliculus.

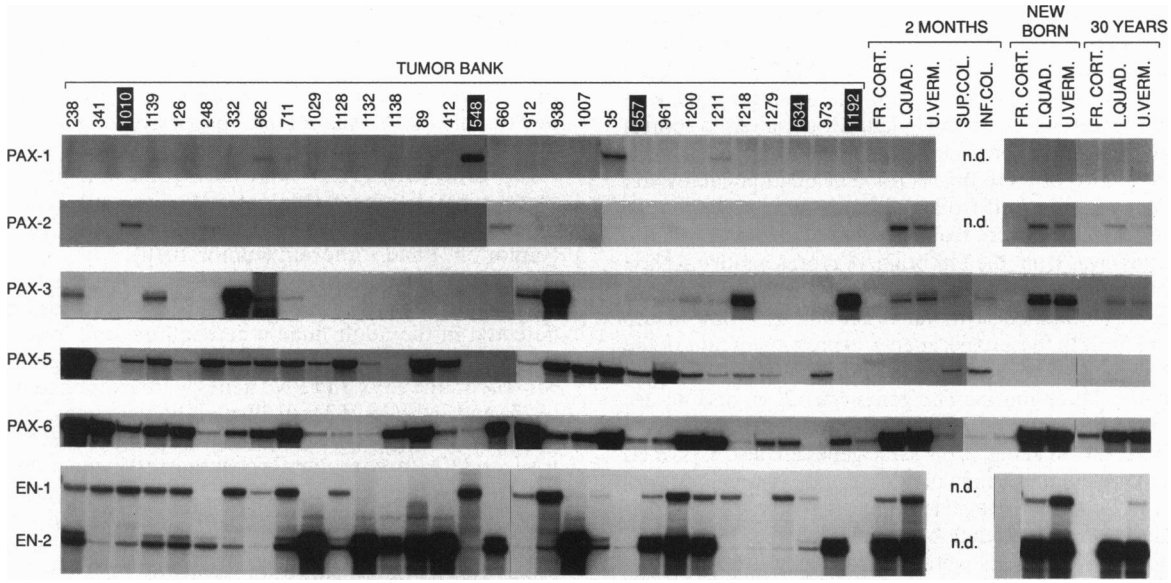

FIG. 2. Expression of *PAX* and *EN* genes in medullablastomas. Total RNA of the same tumors and normal tissues that were analyzed in the experiment of Fig. 1 was subjected to RNase protection by using RNA probes for the different *PAX* and *EN* genes. All autoradiographs were exposed for 2 days, and only the relevant parts containing the RNase-protected signals are shown. Abbreviations are as in Fig. 1. n.d., Not determined.

Contrary to the classical medulloblastomas, analysis of the desmoplastic variant (tumor 341) proved to be more informative. Staining for reticulin fibers revealed the characteristic morphology of desmoplastic medulloblastoma, which consists of areas with intensive reticulin synthesis and of reticulin-free "pale islands." These latter islands contained cells with long processes that expressed NSE (Fig. 3*b*). In contrast, the more densely packed cells of the reticulin-expressing regions failed to express this neuronal marker (Fig. 3*b*). Consistent with the recent analysis of Schiffer *et al.* (19), an inverse expression pattern was seen for the proliferation marker Ki-67, which was synthesized in reticulin-rich areas, but not in pale islands (Fig. 3*d*). Similarly, *PAX5* transcripts could be localized by *in situ* hybridization in the reticulin-expressing regions but not in the pale islands of the tumor (Fig. 3*c*). Expression of *PAX6* was instead detectable in both tumor areas (Fig. 3*e*), and no staining was observed with a control sense RNA probe (Fig. 3*f*). The same results were obtained with three additional desmoplastic medulloblastomas, for which only formalin-fixed paraffin-embedded material was available (data not shown). In summary, we conclude, therefore, that expression of *PAX5*, but not that of *PAX6*, correlates positively with cell proliferation and inversely with neuronal differentiation in desmoplastic medulloblastoma.

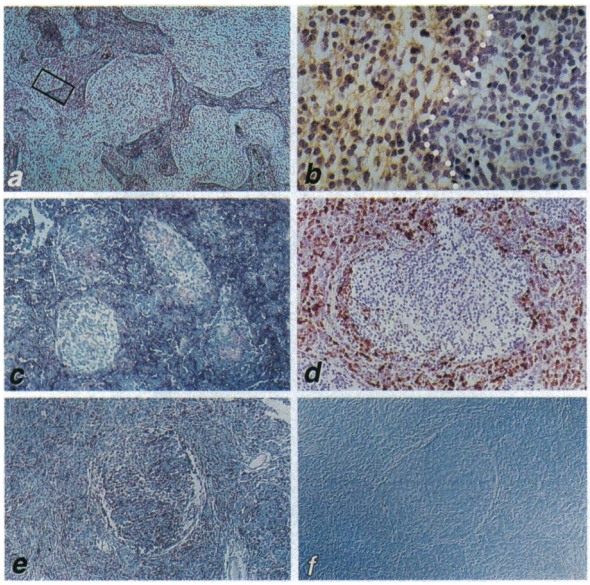

FIG. 3. Expression of *PAX5* and *PAX6* in the desmosplastic variant of medulloblastoma. Analysis of tumor 341. (*a*) Reticulin staining. (×10.) (*b*) NSE staining. A close-up view of a tumor area equivalent to the boxed region in *a* is shown. The dotted line indicates the approximate border between differentiated (to the left) and undifferentiated (to the right) regions. (×100.) (*c*) *In situ* hybridization with the antisense PAX5 RNA probe. (*d*) Staining with the monoclonal antibody MIB-1 recognizing the proliferation marker Ki-76 (19). (*e*) *In situ* hybridization with the antisense PAX6 RNA probe. (*f*) *In situ* hybridization with the control sense PAX6 RNA probe. (*c–f*, ×50.)

DISCUSSION

In this report we have investigated the expression of developmental control genes of the *PAX* and *EN* families in medulloblastoma. Five of the nine mammalian *PAX* genes (*PAX1*, *-2*, *-3*, *-5*, and *-6*) and the two *EN1* and *EN2* genes were expressed, in various combinations, in the different tumors analyzed. The most consistently expressed genes were *PAX5* (70%), *PAX6* (78%), *EN1* (57%), and *EN2* (78% of all cases). All these genes are most likely transcribed in the same tumor cell, since classical medulloblastoma consists of a homogeneous tissue of undifferentiated cells (26).

The expression patterns of the different *Pax* and *En* genes in the CNS have been studied in detail in the mouse. These analyses indicated that the *Pax-2*, *Pax-5*, *En-1*, and *En-2* genes are all initially expressed in the midbrain–hindbrain boundary region of the midgestation embryo that subsequently gives rise to the cerebellum (18, 20, 27, 28). Later in embryogenesis, transcription of the *Pax-2* and *Pax-5* genes (20, 27) is rapidly down-regulated and *En-1* expression is gradually reduced in the developing cerebellum (18). In contrast, strong expression of the *En-2* gene persists in the adult cerebellum (18). The *Pax-6* gene (13, 29) like the *En-2* gene (18) is expressed in the ventricular zone and external granular layer of the developing cerebellum, while *Pax-3* expression is restricted to the ventricular zone only (13). In the adult cerebellum, *Pax-2* is expressed in the Golgi neurons of the granular layer (13), *Pax-3* is expressed in the Bergmann glia and basket cells of the Purkinje cell layer (13), and both *Pax-6* and *En-2* are expressed in the granule neurons of the internal granular

layer (13, 18). In agreement with these findings in the mouse, we have detected expression of *PAX2*, *-3*, and *-6* and *EN2* in the human cerebellum by RNase protection analysis. In addition, we have also observed weak *EN1* expression in the human neonatal and adult cerebellum (Fig. 2).

Do the observed expression patterns of *PAX* and *EN* genes in medulloblastoma provide any insight into the histogenesis of this tumor? It is certainly interesting to note that *PAX6* and *EN2*, which are most consistently expressed in medulloblastoma, are also strongly expressed in the granule neurons present in the internal granular layer of the cerebellum. In contrast, *PAX2* and *PAX3*, which are transcribed in other cell types of the cerebellum, are expressed only rarely in medulloblastoma. Recent cell transplantation experiments demonstrated that the progenitor cells in the external granular layer of the developing mouse cerebellum give rise exclusively to the granule neurons of the internal granular layer (5). Our expression data, therefore, provide molecular evidence to support the hypothesis that the external granular cell layer is the origin of medulloblastoma (3).

PAX1 and *PAX5* are expressed, albeit at different frequencies, in medulloblastomas, and yet these two genes are normally not expressed in the neonatal and adult cerebellum of humans (Fig. 2) and mice (13). The mouse *Pax-1* gene is expressed in the developing vertebral column, and its expression has so far not been reported in any tissue of the CNS (30). The two medulloblastomas and one PNET of the forebrain that express *PAX1* (Fig. 2) provide examples of deregulated expression of *PAX1* in cells of neuroectodermal origin. The consistent expression of *PAX5* in a high proportion (70%) of medulloblastomas can be interpreted in two ways. The undifferentiated cells of medulloblastoma might be blocked in their differentiation capacity at a very early stage of cerebellar development corresponding to the time when *PAX5* is still expressed in the midbrain–hindbrain boundary region of the embryo. An analogous explanation has been put forward to account for elevated *PAX2* and *PAX8* expression in Wilms tumor compared to adult kidney (31, 32). However, we do not favor this interpretation for *PAX5*, as other genes (*Pax-2* and *Wnt-1*), which are normally coexpressed with *Pax-5* in the midbrain–hindbrain junction of the mouse embryo, were expressed vary rarely (*PAX2*) or not at all (*WNT1*) in medulloblastoma (Fig. 2; data not shown). Moreover, an early differentiation block could also not explain the high frequency of *PAX6* expression in medulloblastoma, since *Pax-6* is not yet expressed early on in the midbrain–hindbrain boundary region of the mouse embryo (29). For these reasons, we favor the second possibility that expression of *PAX5* is up-regulated during medulloblastoma formation.

PAX genes can be considered protooncogenes, as their overexpression apparently results in transformation of rat fibroblasts (15) and as chromosomal translocations involving either *PAX3* or *PAX7* are consistently found in alveolar rhabdomyosarcoma (16, 17). In addition, phenotypic analyses of mouse mutants with molecular lesions in individual *Pax* genes have also pointed to a critical role of these transcription factors in the proliferation of progenitor cells of the affected tissues (for review, see refs. 11 and 12). Moreover, inhibition of *PAX5* expression interferes with mitotic stimulation of splenic B cells (33). Thus this evidence implicates *PAX* genes, and *PAX5* in particular, in the control of cell proliferation. The expression pattern of *PAX5*, but not that of *PAX6*, is also compatible with such a role in medulloblastoma. In classical medulloblastoma, both *PAX5* and *PAX6* are uniformly expressed throughout the tumor tissue that mainly consists of undifferentiated cells. In the desmoplastic variant of medulloblastoma, *PAX5* expression is, however, localized to the undifferentiated areas of the reticulin-positive proliferating cells and, in contrast to *PAX6*, is absent in the differentiated reticulin-free islands consisting of quiescent cells (Fig. 3). Hence, *PAX5* expression positively correlates with cell proliferation in desmoplastic medulloblastoma.

In summary, our data demonstrate consistent expression of *PAX5*, *PAX6*, *EN1*, and *EN2* in medulloblastomas. In particular, expression of the *PAX5* gene appears to be up-regulated, which may relate to the genesis of medulloblastoma in two possible ways. Either deregulated expression of *PAX5* is only a consequence of tumor formation or, alternatively, is causally involved in the establishment and/or maintenance of the neoplastic phenotype. The latter hypothesis is clearly amenable to experimental verification by targeting expression of the *PAX5* gene to the appropriate cerebellar compartment in transgenic mice.

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