Expression of SMARCF1, a Truncated Form of SWI1, in Neuroblastoma

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Previously we cloned and mapped a *B120* gene to human chromosome 1p35-36.1 where possible suppressor genes for various neuroendocrine tumors including neuroblastoma have been mapped. Very recently, *B120* was identified as a truncated form of *p270*, a putative human counterpart of SWI1. In the present study, expression of the *B120* gene product was immunohistochemically investigated in 23 neuroblastomas. We also examined *B120* expression in neural stem cells in developing brain and intact adrenal medulla. Four of 23 neuroblastomas strongly expressed *B120* gene product in both cytoplasm and nucleus. The other neuroblastomas expressed *B120* gene product in the nucleus; however, the intensity of staining was much weaker and equivalent to that in developing human brain stem cells in the subventricular region. *B120* gene product was less strongly expressed in intact adrenal medulla. Subsequently, we performed loss of heterozygosity studies on 19 neuroblastomas using the polymorphic markers D1S195 and D1S511 located near the *B120* gene. Loss of heterozygosity was observed in three of 19 tumors that abundantly expressed B120 protein. Furthermore, neuroblastoma cells were transfected with *B120* expression vector. These transfected neuroblastoma cells adhered to each other and aggregated. Differential display experiments followed by reverse transcriptase-polymerase chain reaction and Northern blot analysis were performed and three molecules with altered expression in *B120*-transfected neuroblastoma cells were identified. One of three genes seemed to be a proliferation-related and cell cycle-related nucleolar protein, p120, encoding gene. We further characterized the genomic structure of *B120*. *B120* appeared to be encoded by 17 exons in more than 20-kbp genomic DNA. The present findings contribute to understanding of the *B120* gene, a truncated form of human *SWI1*, an approved term for which is *SMARCF1*, in normal cells and neuroblastomas. (*Am J Pathol 2001, 158:663–672*)

We previously reported a novel human gene designated *B120*, which was strongly expressed in neural cells, muscles, and thymus.[1] The deduced amino acid sequence of *B120* included a novel DNA-binding region, termed ARID (AT-rich interactive domain). We also mapped the *B120* gene to 1p35-36.1,[2] in which the putative tumor suppressor gene for neuroendocrine tumors including neuroblastoma is located.[3,4] Caron and colleagues[5] hypothesized that at least two tumor suppressor genes are located within 1p35-36: an imprinted distal 1p36 locus and a nonimprinted 1p35-36.1 locus. Various studies have indicated that these genes are affected in at least one third of primary neuroblastomas.[6] Surprisingly and very interestingly, the human counterpart of SWI1, termed *p270*, was recently identified and appeared to be partly identical to *B120* cDNA.[7] However, *B120* differed from *p270* at the 5′ and 3′ termini and encoded a truncated form of p270 protein.[1,7] The Nomenclature Committee authorized by the Human Genome Organization recommended that we use the approved symbol *SMARCF1* (SWI/SNF-related, matrix associated, actin-dependent regulator of chromatin, subfamily f, member 1) for the human counterpart of *SWI1*, instead of *B120* and *p270*. The SWI/SNF complex is a 2-mDa multisubunit DNA-dependent ATPase that contributes to the regulation of gene transcription by altering chromatin structure.[8,9] An increasing number of reports have described the important roles played by the SWI/SNF complex in gene regulation, including the cell cycle and tumorigenicity.[10] Versteege and colleagues[11] found that a member of the SWI/SNF chromatin-remodeling complex (*hSNF5/INI1*) was mutated in aggressive pediatric cancer. Taylor and colleagues[12] also identified familial posterior fossa brain tumors, including primitive neuroectodermal tumors, with germline mutation of the *hSNF5* gene. These recent findings led us to examine the expression and role of B120 protein in neuroblastoma.

Materials and Methods

Patients and Tissue Samples

The participants in this study were 23 patients with neuroblastoma who were selected by experienced patholo-

gists and classified by clinical and pathological diagnosis. The main clinical characteristics of the relevant patients are summarized in Table 1. Tissues from patients and spontaneously aborted fetuses were routinely formalin-fixed and embedded in paraffin. Normal adrenal glands were surgically resected from patients with renal cancer. In several cases, adrenal glands resected by autopsy were also used. The Committee of Kochi Medical School approved the protocol for this study. Tissues were routinely formalin-fixed and paraffin-embedded.

Immunohistochemical Staining

Details of the procedures used including the characterization of a monoclonal antibody to B120 protein termed 39-7-7, were previously described.[1] Briefly, we immunized mice with recombinant gene product that was produced in *Escherichia coli* cells using the T7 polymerase-dependent expression system, as described by Studier and Moffatt.[13] We produced a recombinant protein of ~600 bp from *B120* cDNA in *E. coli* cells. This recombinant protein, with an approximate molecular weight of 30 kd, corresponded to the C-terminal part of B120 protein. After the third immunization, the splenocytes were fused with P3X63Ag8U.1 cells. After screening, one hybridoma clone was found to produce antibody useful even in the routinely processed formalin-fixed and paraffin-embedded tissue sections. We also used isotype-matched monoclonal antibody to *Trichosporon beigelii*, a fungus, as a negative control. A previous study revealed that the control monoclonal antibody did not react with various human tissues.[14] The tissue sections were cut, deparaffinized, preincubated with fetal bovine serum, and incubated with 39-7-7 culture supernatant for 1 hour at room temperature. After washing with phosphate-buffered saline (PBS), the sections were stained using a streptavidin-biotin complex peroxidase kit (DAKO LSAB kit; Dakopatts, Tokyo, Japan). The procedures were performed according to the manufacturer's protocol. Finally, reaction was developed with diaminobenzidine. The tissue sections were counterstained with hematoxylin or methyl green. Based on the number of positively stained neuroblastoma cells, the immunohistochemical staining was semiquantitatively scored as weakly positive (>5 to 20%), moderately positive (>20 to 80%), or markedly positive (>80%).

Cells and Cell Culture

Three neuroblastoma cell lines, IMR-32, TGW, and NH-12, were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Cell Culture Laboratories, Cleveland, OH) and 50 μg/ml gentamicin (Life Technologies, Grand Island, NY). Previous reports from other laboratories revealed the N-*myc* amplication in IMR-32 and TGW cells.[15,16]

Western Immunoblotting

Immunoblotting was performed essentially according to the method of Towbin and colleagues.[17] The procedure was described in detail previously. Briefly, cultured cells or tissues were homogenized in a lysis buffer (20 mmol/L Tris-HCl, pH 7.4, containing 0.5% Nonidet P-40, 1 mmol/L phenylmethyl sulfonyl fluoride, 5 μg/ml aprotinin). The protein concentration of each lysate was determined and used to load an equal amount of protein per well (5μ g/well) of a sodium dodecyl sulfate (SDS)-polyacrylamide gel. These separated proteins were transferred to Immobilon polyvinylidene difluoride sheets (Millipore Corp., Bedford, MA). The membrane was blocked with PBS and 10% horse serum, incubated with monoclonal antibodies, followed by incubation with goat F(ab')$_2$ anti-mouse immunoglobulins conjugated with peroxidase (Biosource International, Camarillo, CA). We used isotype-matched monoclonal antibody as a negative control. Finally, the reaction was developed with diaminobenzidine.

Screening and Characterization of Genomic DNA Clones

The screening was performed on a human genomic P1 library obtained from Genome Systems by polymerase chain reaction (PCR) with previously reported primers K1 to K6[1] using an long and accurate (LA) PCR kit (Takara Shuzo, Ohtsu, Japan). PCR conditions were previously reported.[1] Briefly, genomic P1 clones were amplified by an initial denaturation step of 5 minutes at 95°C followed by 30 cycles of two-step PCR (20 seconds at 98°C and 5 minutes at 68°C) in the presence of 1.5 mmol/L of MgCl$_2$. Positive P1 clones were isolated and subcloned into pBluescript SKII (Stratagene, La Jolla, CA). Synthetic oligonucleotides were used to sequence both strands of double-stranded plasmid DNA with an Automated Laser Fluorescent ALF sequencer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The sizes of the introns were determined either by sequencing or by long-range PCR using a LA-PCR kit according to the manufacturer's instructions. We also confirmed genomic DNA structure from healthy volunteers by long-range PCR using synthesized primers.

Radiation Hybrid Mapping

The whole radiation hybrid GeneBridge 4 panel (GB4) was used (Research Genetics, Inc., Huntsville, Alabama). It is composed of 93 human-on-hamster somatic cell lines.[18] We performed PCR with K1 to K6 primers as described above. PCR products were electrophoresed on 1.5% agarose ET-Br gels. The PCR reaction was performed twice. The logarithm odds (LOD) scores were processed with the RHMAPPER program on the Whitehead Institute/MIT center server (URL: http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl), with a required LOD score for linkage equal to 15.[19]

Table 1. Stage and Sex of Patients, Semiquantitative Score of B120 Protein Expression, and LOH on the B120 Gene Region Flanked by D1S511 and D1S195 Polymorphic Marker at 1p35-36.1

Clinical stage, number of cases (male/female)	B120 expression (male/female)	LOH at B120 gene region (male/female)*
Stage I, 6 cases (3/3)	Weakly, 1 case (0/1) moderately, 4 cases (2/2) markedly, 1 case (1/0)	LOH(−), 5 cases (2/3) LOH(+), 1 case (1/0)
II, 5 cases (3/2)	Weakly, 1 case (1/0) moderately, 4 cases (2/2)	LOH(−), 3 cases (2/1)
III, 4 cases (1/3)	Moderately, 2 cases (1/1) markedly, 2 cases (0/2)	LOH(−), 2 cases (1/1) LOH(+), 1 case (0/1)
IV, 6 cases (3/3)	Weakly, 1 case (0/1) moderately, 4 cases (2/2) markedly, 1 case (1/0)	LOH(−), 4 cases (2/2) LOH(+), 1 case (1/0)
IVs, 2 cases (1/1)	Moderately, 2 cases (1/1)	LOH(−), 2 cases (1/1)

*Four cases were not available for LOH analysis.

Loss of Heterozygosity (LOH) Analysis

DNA was extracted from both the tumor-rich and tumor-free areas of tissue sections using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA) in accordance with the manufacturer's protocol. We selected tumor-rich specimens in which the proportion of neuroblastoma cells in the tumor component was estimated to be at least 80%. Extracted DNA was examined for genetic alterations using the polymorphic markers D1S511 and D1S195. Both markers contain (CA)n repeats around the B120 gene locus. D1S511 and D1S195 were amplified with primer sets of 5′-tctaatttttcagcaagtgccacag-3′ (sense) and 5′-cagtgagccaagattgtgccaatgc-3′ (antisense); 5′-taaagaggaaaggtggacggtggg-3′ (sense) and 5′-attcctcttgagcatcaccccagc-3′ (antisense), respectively. For each primer pair, a sense primer only was fluorescently labeled at the 5′ end. Template genomic DNAs (100 ng) were amplified by PCR using a thermal cycler (Takara Co. Ltd., Ohtsu, Japan) in a volume of 50 μl containing 1.5 mmol/L of Mg Cl₂, 200 μmol/L of each dNTP, 50 pmol of each primer, and 2.5 U of Taq polymerase (Perkin Elmer, Norwalk, CT). The PCR consisted of 1) initial denaturation and activation of Taq polymerase at 95°C for 2 minutes; 2) 30 cycles of 95°C for 30 seconds, 58°C (D1S195) or 60°C (D1S511) for 45 seconds, and 72°C for 60 seconds. PCR products were run and analyzed using an automated fluorescent DNA sequencer on a 6% polyacrylamide denaturing gel. Allele ratios were calculated as principally described by Cawkwell and colleagues.[20] Because incomplete losses are commonly observed and may reflect either normal cell contamination or tumor heterogeneity, a ratio of ≤0.50 was considered indicative of LOH.

Expression Plasmids and Stable Transfection

The construction and stable transfection of a pCI-neo expression vector containing the B120 cDNA (pCI-neo-B120) was previously reported.[1,2] Briefly, a human B120 full-length cDNA was prepared and transfected into IMR-32, TGW, and NH-12 cells using DOTAP transfection reagent (Boehringer Mannheim GmbH, Mannheim, Germany) in accordance with the manufacture's protocol. As a negative control, the pCI-neo expression vector alone was introduced into cells in parallel. Permanent *B120*-transfected and pCI-neo expression vector alone-trans-

fected cell lines were established after G418 selection (Sigma, St. Louis, MO) and maintained with the medium as used for the original cell lines.

Differential Display of mRNA (DDRT-PCR) and Subsequent RT-PCR

Total RNAs were extracted from *B120*-transfected and control vector alone-transfected IMR-32 neuroblastoma cells. Then RNAs were treated with DNase I for 10 minutes at 37°C. One μg of each RNA was reverse-transcribed with 200 U of Superscript II reverse transcriptase (Life Technologies, Inc.) in the presence of 50 pmol of T12MN primers, where M represents A, C, or G and N is T, A, C, or G, in 20 μl of RT buffer (25 mmol/L Tris, pH 8.3, 37.5 mmol/L KCl, 1.5 mmol/L MgCl₂, 10 mmol/L dithiothreitol, 20 μmol/L dNTP). After heat inactivation of the reverse transcriptase at 95°C for 5 minutes, subsequent PCR amplification was performed using one-tenth of the cDNA with 50 pmol of the appropriate T12MN primer in combination with 10 pmol of arbitrary 10-mer primer. The PCR reaction was performed with 2 U of *Taq* polymerase and 0.075 μl [α-³²P]dATP (1,000 Ci/mmol) in 20 μl of PCR buffer (10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgC12, 2 μmol/L dNTP). The cycling parameters were as follows: 94°C for 4 minutes, and then 40 cycles of denaturation (94°C for 30 seconds), annealing (42°C for 2 minutes), and elongation (72°C for 30 seconds), followed by a 72°C elongation step for 7 minutes. The amplified cDNAs were separated on a 6% sequencing gel. Differentially displayed cDNAs were excised from dried sequencing gel, boiled in 100 μl of distilled water for 15 minutes, precipitated with ethanol, and suspended in 10 μl of water. After re-amplification by PCR with the identical primer set, amplified PCR products were subcloned into pGEM-T Easy vector (Promega Corporation, Madison, WI). Finally, the PCR products in pGEM-T easy plasmid were transfected into JM109, and sequenced. We detected three partial DNAs with increased expression in *B120*-transfected cells. To determine whether these genes were more strongly expressed in *B120*-transfected neuroblastoma cells than in control cells, we performed reverse transcriptase (RT)-PCR. Total cellular RNA was prepared from cells using RNA-zol B (Biotex Laboratory, Houston, TX). We used 1 μg of each total RNA as substrate for cDNA
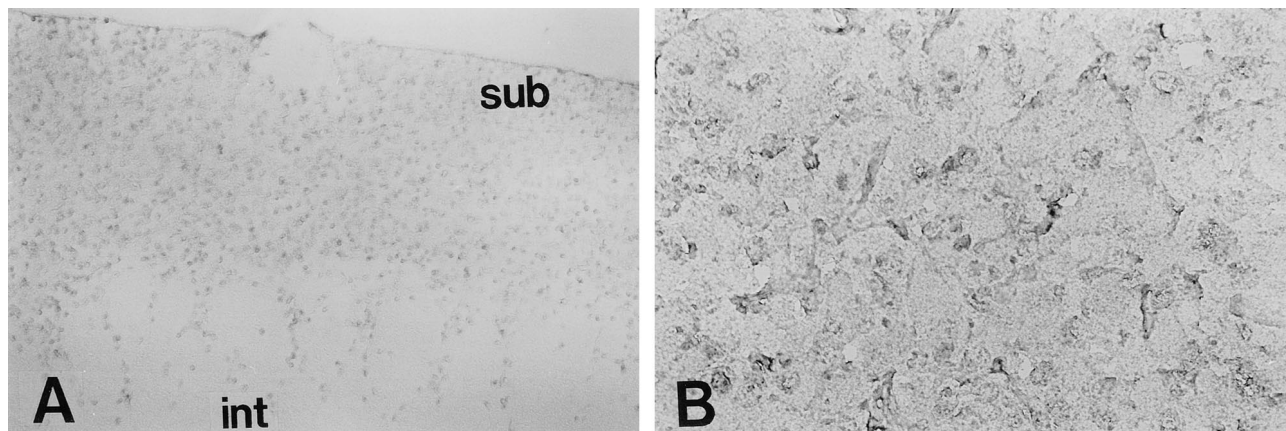
Figure 1. Immunohistochemical staining with 39-7-7 antibody, which was generated and specifically reacted with a the C-terminal part of B120 protein, in human developing brain (**A**) and intact adrenal medulla (**B**). Immunohistochemical staining of developing precentral gyrus of cerebrum at 17 weeks, the neuroprogenitor cells in the germinal zone at subventricular area (sub) was stained by 39-7-7. Migrating neural cells in the intermediate layer (int) were not stained by 39-7-7 antibody. The pericytoplasmic region in pheochromocytes in intact adrenal gland medulla was also stained, but not as strongly by 39-7-7 antibody. Original magnifications: ×100 (**A**), ×400 (**B**).

synthesis with the RT-PCR kit (Life Technologies, Gaithersburg, MD). The cDNA synthesis and following PCR was performed according to the manufacture's instructions. RT-PCR detection of mRNAs of *p120*, *B120ind1*, and *B120ind2* was performed using the newly synthesized primer sets as follows below: 5′-cggaccaatacccttgaaaacccgacgc-3′ and 5′-cgctcagcattggcgtcattggcaag-3′ for *p120* gene. 5′-catgagttcatgaaaagatattaggg-3′ and 5′-gagctgagggctagccagggagg-3′ for *B120ind1*. 5′-ccctgacagcctgtactgctgggtccag-3′ and 5′-ggcaataccgggtaacctattaggcc-3′ for *B120ind2*. We also used *G3PDH* primers, 5′-tccaccaccctgttgctgta-3′ and 5′-accacagtccatgccatcac-3′.

Northern Blot Analysis

Multiple-tissue Northern blots (Clontech, Palo Alto, CA) were screened with an ~1-kb cDNA fragment spanning the 3′ terminus of the coding sequence for *B120* (nucleotides 3-kb to 4-kb). The probe was radiolabeled with [α-^{32}P]dCTP by random-primed labeling kits (Boehringer Mannheim). Membranes were washed in 2× standard saline citrate (SSC)/0.1% SDS at 55°C, and then with 0.1× SSC/0.1% SDS at 55°C. We also examined *p120* gene expression in *B120*-transfected neuroblastoma cells by Northern blot. Briefly, 20 μg of total RNA from TGW neuroblastoma cells transfected with pCI-neo-*B120* or pCI-neo alone were loaded onto a denatured agarose gel. A PCR-amplified *p120* cDNA that covered the coding region was radiolabeled and used as a probe. Membranes were washed in 2× SSC/0.1% SDS at 55°C, and then with 0.1× SSC/0.1% SDS at 60°C.

Results

### Immunohistochemical Staining

We immunostained tissues from patients with neuroblastoma, intact adrenal gland, and human developing brain.

Neuroprogenitor cells in fetal germinal zone at 17 and 18 weeks demonstrated staining by anti-B120 monoclonal antibody (Figure 1A).

Neural cells migrating to the cortex in the intermediate zone were not significantly stained by anti-B120 antibody. In normal adrenal gland medulla, staining was less strongly observed (Figure 1B). Although these normal cells were composed of heterogenetic clones, the semiquantitative score was equivalent to weakly positive for all normal tissues examined. In contrast to these normal counterparts, four of 23 neuroblastomas tested exhibited intense staining (scored as markedly positive) in both nucleus and cytoplasm by anti-B120 antibody (Figure 2A); however, no significant reactivity was observed with control isotype-matched monoclonal antibody (Figure 2B). The staining intensities of 16 and three specimens tested were semiquantitatively scored as, respectively, moderate (>20 to 80%) and weak (>5 to ~20) positive. Staining in these 19 cases was restricted to the nucleus, as shown in Figure 2C. The staining results were not related to patient's age and sex. The results are also summarized in Table 1.

Western Immunoblotting

We also performed immunoblotting to determine whether neuroblastoma cells expressed the B120 molecule. A single band with a molecular weight of ~120 kd was observed in the Nonidet P-40 soluble fraction of IMR-32 neuroblastoma cells as demonstrated in Figure 3 (lane D). As expected, a similar but stronger band was observed for B120-transfected IMR-32 cells (lane B). However, no significant reactivity was observed in Nonidet P-40-extracted human developing brain at 17 or 18 weeks with the 39-7-7 antibody (lanes A and C, respectively). No significant band was observed with control isotype-matched antibody in IMR-32 cells (lane E).
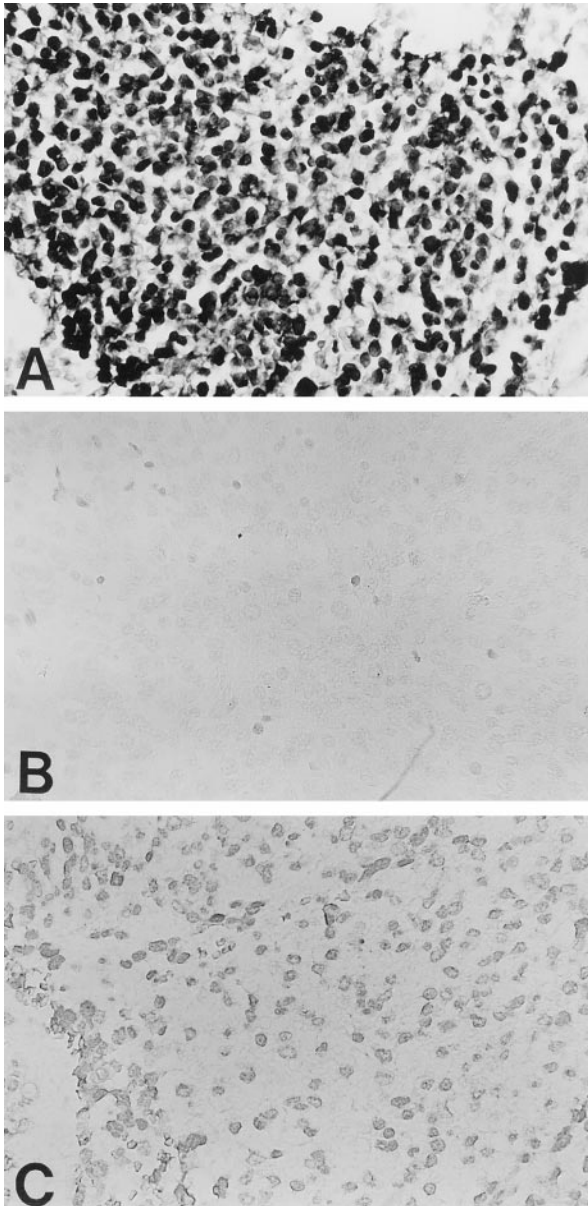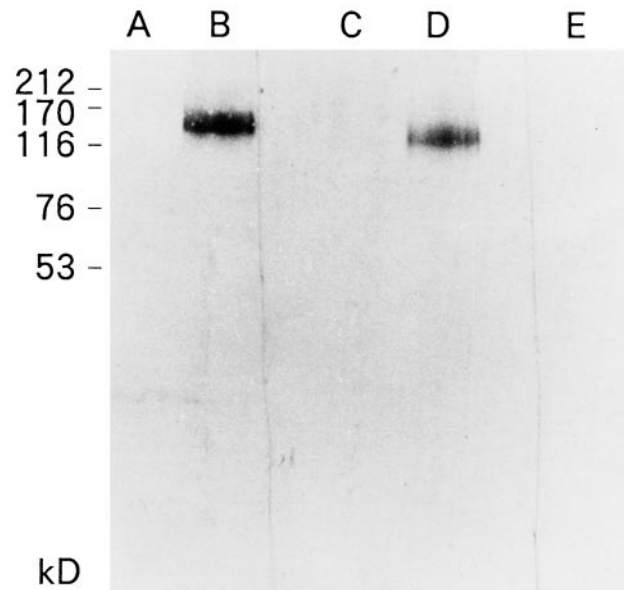
Figure 2. Neuroblastoma cells were strongly stained by 39-7-7 (**A**) but not with control isotype-matched monoclonal antibody (**B**). Not only the nucleus of neuroblastoma cells but also the cytoplasm were strongly stained by 39-7-7 (**A**). Representative moderately positive staining of neuroblastomas with 39-7-7 antibody (**C**). Original magnifications, ×400.


Figure 3. Western blot analysis of the 0.5% solubilized fraction of IMR-32 neuroblastoma cells and fetal brain with 39-7-7 antibody and control antibody. **A:** Fetal-developing brain extracts at 17 weeks. **B:** *B120*-transfected IMR-32 cells. **C:** Fetal-developing brain extracts at 18 weeks. **D** and **E:** IMR-32 cells. **Lanes A**, **B**, **C**, and **D** were reacted with 39-7-7. **Lane E** was reacted with control antibody. A single band with an approximate molecular weight of 120 kd was detected in the IMR-32 neuroblastoma cells with 39-7-7 antibody (**lane D**). In contrast, no band was observed with isotype-matched control antibody (**lane E**). A single strong band with an approximate molecular weight of 120 kd was also detected in *B120*-transfected IMR-32 cells with 39-7-7 antibody (**lane B**). No significant band was observed in fetal developing brain extracts at 17 and 18 weeks (**lanes A** and **C**, respectively).

Radiation Hybrid Mapping

The *B120* gene was previously localized by a fluorescence *in situ* hybridization study to 1p35-p36.1. To determine more precisely the position of the *B120* gene, we performed radiation hybrid mapping. The *B120* gene was localized at 1.71 cR from WI-9232 on chromosome 1p. It is flanked by the polymorphic markers D1S511 and D1S195, corresponding to a 8-cM interval.

LOH Analysis

Four of 23 samples were not analyzed in this study. Two cases were not informative and two other cases could not be investigated for inadequacy of extracted DNA. LOH at both D1S511 and D1S195 regions was detected in three *B120*-overexpressed neuroblastoma specimens (Figure 4). In contrast, LOH at neither the D1S511 nor the D1S195 region was found in 14 neuroblastomas in which the staining score was moderately to weakly positive and staining signals with anti-B120 antibody were restricted to the nucleus. LOH was not detected at one of two markers in the other two non-*B120* overexpressed neuroblastomas; however, another marker was not informative by homozygous. We considered these two neuroblastomas as LOH (−) in this study. These results were also summarized in Table 1.

Determination of B120 Genomic Structure

Three P1 DNA fragments were characterized by screening. One of the three was found to have the full-length genomic structure of *B120* by PCR with the 5′- or 3′-terminal region. The other two were found to have a shorter genomic structure. Comparison with the *B120* cDNA confirmed the sequence and showed that the *B120* gene contains 17 exons (GenBank accession numbers: AB024059-75), as illustrated in Figure 5. The DNA sequence of the intron-exon boundaries of the *B120* gene is shown in Table 2. We also confirmed the *B120* genomic structure using genomic DNA obtained from healthy volunteers with primers for intron-exon junctions.
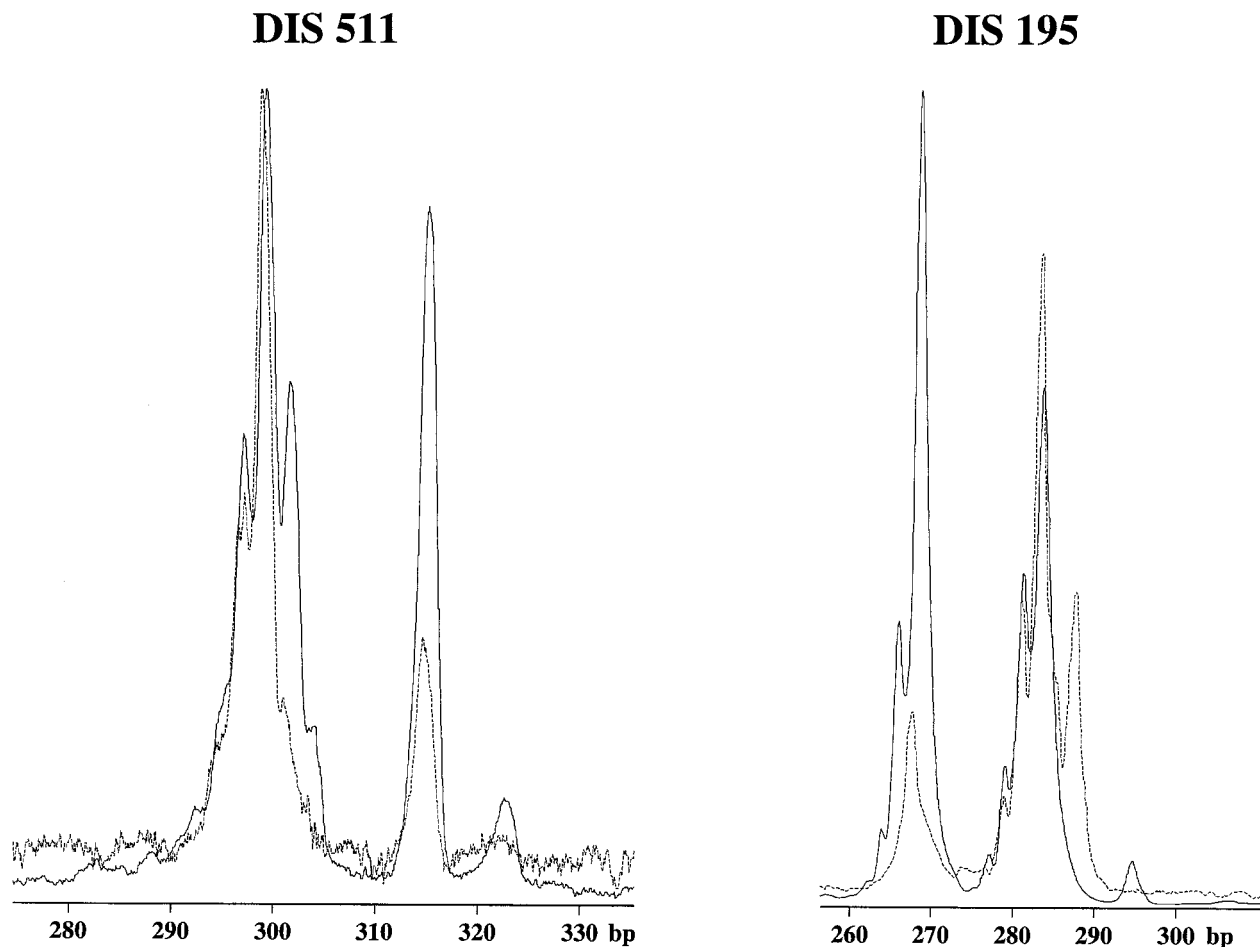
DIS 511

DIS 195

Figure 4. Representative examples of a clonal deletion of D1S511 and D1S195 region in a *B120*-overexpressing neuroblastoma (**broken lines**). For comparison, the genotypes derived from tumor-free areas of an identical patient are superimposed (**solid lines**).

Transfection of *B120* with expression vector into neuroblastoma cell lines TGW, IMR-32, and NH-12 cells were successfully transfected with pCI-neo-*B120* after G418 selection. Control pCI-neo alone-transfected cells exhibited no morphological changes (Figure 6A). *B120*-transfected cells adhered to each other and formed small clusters, as demonstrated in Figure 6B. At the time of passage, these *B120*-transfected cells were separated from each other using trypsin; however, ~6 hours later, cells were again adherent to each other and forming small clusters. Twenty-four hours after trypsinization, small clusters of *B120*-transfected cells were further adhering to other clusters to form larger clusters. These clusters were not completely round or oval and were spike-shaped on their surfaces.
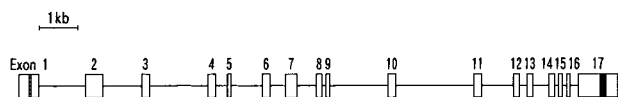
Figure 5. Structure of *B120* gene. The DNA sequences of the intron-exon boundaries are demonstrated in Table 1.

Differential Display of mRNA (DDRT-PCR), Subsequent RT-PCR, and Northern Blot Analysis

We detected several bands indicating overexpression of the respective genes in *B120*-transfected IMR-32 cells. Interestingly, we found no significant bands representing differentially down-regulated after ectopic *B120* expression. Finally, three representative partial cDNAs were sequenced. A BLAST homology search revealed that one of the three is identical to a previously reported p120 nucleolar protein encoding cDNA. The other two appeared to be novel genes, and were temporarily designated *B120ind1* and *B120ind2* (GenBank accession numbers. AB032363 and AB032364, respectively). Very recently, *B120ind1* and *B120ind2* were found apparently to be a part of Homo sapiens clones RP11-56A1 and chromosome 5 clone CTB-11I22 (GenBank accession numbers: AC007482 and AC011376), respectively. Both RP11-56A1 and CTB-11I22 clones were sequenced by the Human Genome Project without any functional study. Primer sets were synthesized as described in Materials and Methods to detect each mRNA in transfected or

Table 2. DNA Sequence of the Intron-Exon Boundaries of *B120* Gene

(Exon 1) tatacacagcag-gtagatggtgattgt------tttcctttcacacag-**attcctccttatgga**
(Exon 2) cctccaccgcag-gtaagatatccctgc-----tttttcttgttgttag-**gagctatctcaagat**
(Exon 3) tccagcttgcct-gtgagtatttctgca-----atatatatgttctag-**gatctatctggttca**
(Exon 4) tgcagtgccag-gtaccctcaagtgct-----ttgtctggagcag-**gcaaccagatgcca**
(Exon 5) ccaagatcgag-gtgagagcctgggt-----acccacaaatag-**gttatatgcagagga**
(Exon 6) tggcccacaag-gtcagtatactaccc-----ctcccctcccag-**gtggctaccccaggc**
(Exon 7) tccaaaacag-gtaaggcctgggaag-----catcttcttccttag-**gccgccaggctacc**
(Exon 8) aattctgcag-gtaagtgctagtcat-----tttctcttttacag-**ggatggcagccagcc**
(Exon 9) aaatccaag-gtagtgattttttgtc------tgaaccttccag-**aaatccagttcttctac**
(Exon 10) attgactcag-gtgagtgggcgcct-----ttggttatacag-**gtcaacaagaacaaaa**
(Exon 11) cctctcctg-gtaaggatggggtca-----tctgtccacag-**cgggatcaggatctatg**
(Exon 12) ggcatgag-gtaaggccaagagca-----ttcactctggag-**caggagcaattcagttg**
(Exon 13) gaggaaag-gtgactgatctgatt-----ttctacgctcag-**ctccagggagtgatccct**
(Exon 14) agagtgag-gtaagcatgacccca-----aattttgtttag-**gacggagcctggaatagg**
(Exon 15) cagcaacg-gtgagtaaagcctgg-----cctcctatag-**acatgattcctatgg caatc**
(Exon 16) caacagcag-gtgaggagggtagct-----ctctgccttag-**aattacaagcggccaatgg**
Exon 17

original cells. RT-PCR demonstrated that these three genes were expressed more strongly in *B120*-transfected TGW cells than in the original cells (Figures 7 and 8). In our experimental conditions, we detected *B120ind1* and *B120ind2* bands in *B120*-transfected cells after, respectively, 30 and 20 cycles. However, even after 30 cycles we detected no bands with *B120ind1* and *B120ind2* primer sets from control pCI-neo alone-transfected cells (Figure 8). The difference in *p120* gene expression be-
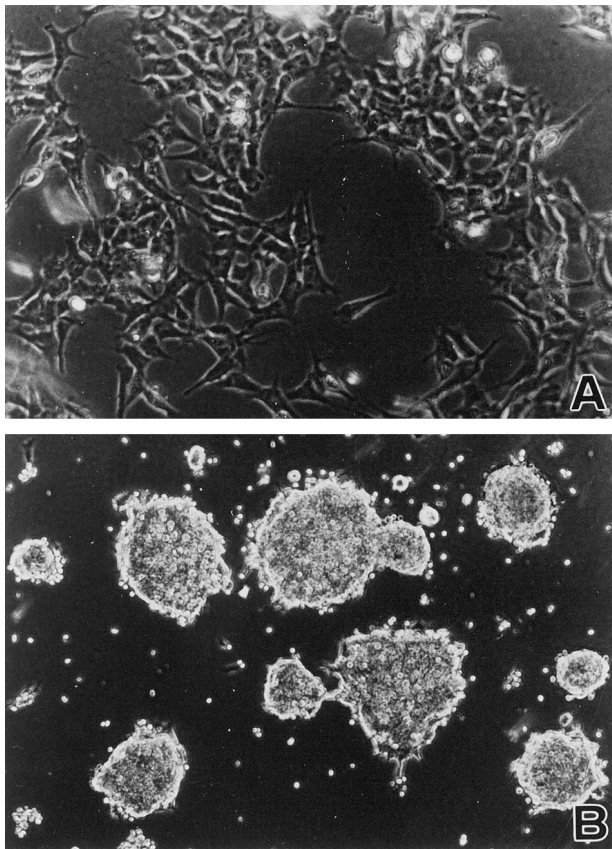
Figure 6. Transfection of *B120* cDNA with expression vector into IMR-32 neuroblastoma cells. *B120*-transfected IMR-32 neuroblastoma cells adhered to each other and formed small clusters (**B**). In contrast, no significant change was observed in control plasmid alone-transfected IMR-32 cells (**A**).
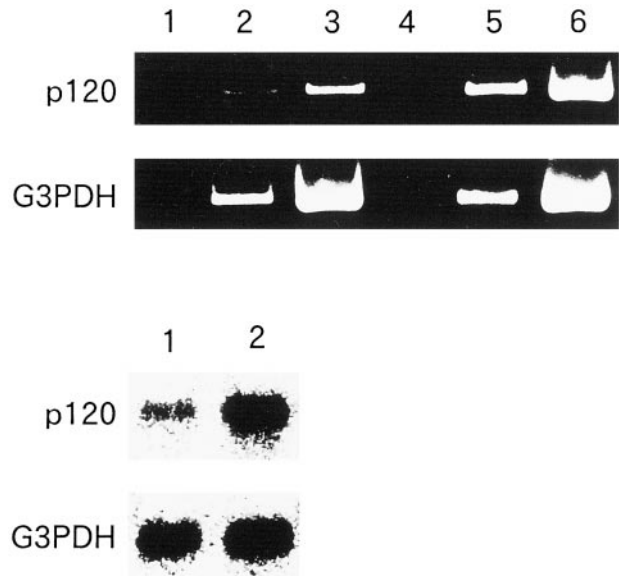
Figure 7. RT-PCR and Northern blot of *B120*-transfected TGW neuroblastoma cells. **Top:** The plasmid alone-transfected TGW cells were applied to RT-PCR (**lane 1**, 10× cycles; **lane 2**, 15× cycles; **lane 3**, ×25 cycles of PCR after RT reaction) with *p120* or *G3PDH* primer sets. *B120*-transfected cells were also examined by RT-PCR (**lane 4**, 10× cycles; **lane 5**, 15× cycles; **lane 6**, 25× cycles of PCR after RT reaction). Overexpression of *p120* in *B120*-transfected TGW cells was demonstrated by RT-PCR. Note the even intensity of internal control bands of *G3PDH* in both *B120*-transfected and plasmid alone-transfected TGW cells. **Bottom:** Northern blot analysis of plasmid alone-transfected and *B120*-transfected TGW cells (**lanes 1** and **2**, respectively). Northern blot analysis also demonstrated the overexpression of *p120* gene in *B120*-transfected cells. Note the even intensity of *G3PDH* band in **lanes 1** and **2**.
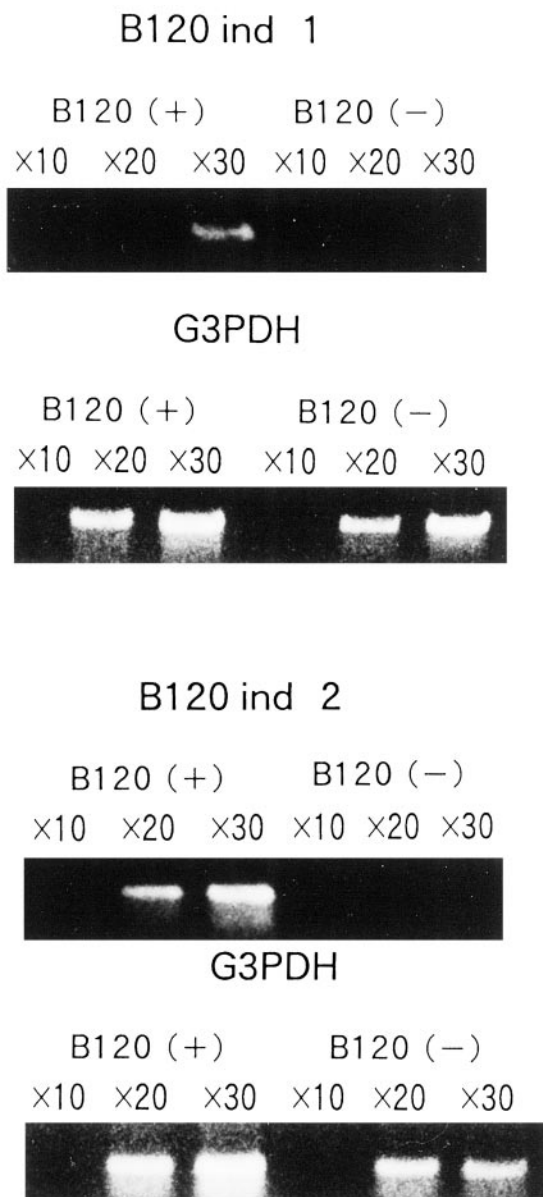
tween the original and *B120*-transfected cells was relatively small (Figure 7). We detected *p120* bands after 15 cycles and 25 cycles in *B120*-transfected and control cells, respectively. Northern blot analysis also confirmed that *B120*-transfected TGW cells expressed much more *p120* than pCI-neo-alone transfected cells.

mRNA Expression in Various Human Tissues

The results are demonstrated in Figure 9. A RNA species migrating at ~8.0 kbp was detected in almost all tissues examined. In addition, we observed other bands at ~5 kbp and 4 kbp in human brain. This 5-kb RNA band was also detected in spleen, thymus, prostate, and colon.

Discussion

B120 was first recognized in a search for genes containing multiple CAG repeats.[1] The *B120* gene was subsequently localized to 1p35-36.1,[2] in which a putative tumor suppressor gene for neuroendocrine tumors including neuroblastomas is located.[4] We previously transfected C3H/10T1/2 fibroblastic cells with *B120* expression vector and observed abundant cytoplasmic lipid deposits and a tiny nucleolus.[3] It seemed unlikely that B120 protein was directly involved in lipid metabolism, because the deduced amino acid sequence for *B120* exhibited no significant homology with previously identified enzymes involved in lipid metabolism. We speculated that the

B120 ind 1

B120 (+)   B120 (−)
×10 ×20 ×30 ×10 ×20 ×30

G3PDH

B120 (+)   B120 (−)
×10 ×20 ×30 ×10 ×20 ×30

B120 ind 2

B120 (+)   B120 (−)
×10 ×20 ×30 ×10 ×20 ×30

G3PDH

B120 (+)   B120 (−)
×10 ×20 ×30 ×10 ×20 ×30

Figure 8. RT-PCR analysis of *B120*-transfected TGW neuroblastoma cells to examine the overexpression of *B120ind1* and *B120ind2*. Overexpression of *B120ind1* and *B120ind2* genes in *B120*-transfected TGW cells. 10×, 20×, and 30× indicate PCR cycles followed by RT reaction. Note the even intensity of internal control bands of G3PDH in both *B120*-transfected and plasmid alone-transfected TGW cells.

B120 molecule might act as a transcriptional factor and increase or induce the expression of other genes involved in lipid metabolism and organization of the tiny nucleolus.[2] Notably, *B120*-transfected fibroblastic cells did not aggregate with each other, as did *B120*-transfected neuroblastoma cells in the present study. The abundant lipid deposits found in *B120*-transfected fibroblastic cells were not observed in *B120*-transfected neuroblastoma cells.

Very recently, Dallas and colleagues[7] identified human SWI/SNF complex protein p270. Surprisingly *p270*, a putative human counterpart of *SWI1*, had an identical portion of B120 cDNA product. The Human Gene Nomenclature Committee recommended an approved symbol

SMARCF1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily f, member 1) for the human counterpart of *SWI1*. The SWI/SNF complex plays fundamental roles in the regulation of gene expression during cell growth, the cell cycle, and development in various organs.[21] Moreover, Versteege and colleagues[11] found that a member of the SWI/SNF chromatin remodeling complex, the hSNF encoding gene, was mutated in aggressive pediatric malignant rhabdoid tumors. Very recently, Taylor and colleagues[12] also found germline mutation of the *hSNF* gene in a family afflicted over multiple generations with posterior fossa tumors of infancy, including the central nervous system (a subset of primitive neuroectodermal tumors).

In the present study, we observed abundant expression of the *B120* gene product in neuroblastomas which exhibited LOH in the *B120* gene region flanked by D1S511 and D1S195 polymorphic marker containing a (CA)n repeat (Figures 2 and 4). Both the cytoplasm and nucleus demonstrated strong positive reaction with anti-B120 monoclonal antibody in these cases, as demonstrated in Figure 2A. We also detected staining in fetal neural stem cells in the germinal zone; however, the staining intensity of normal neuroprogenitor cells was equal to those semiquantitatively scored as weakly positive and staining was restricted to the nucleus (Figure 1). Interestingly, positive signals of B120 staining in non-*B120*-overexpressing neuroblastomas was also restricted to the nucleus and much weaker than that in *B-120*-overexpressing neuroblastomas (Figure 2C). LOH of the *B120* gene region, ie, D1S511 and/or D1S195, was not found in the non-*B120*-overexpressing neuroblastomas tested.

Although we could not directly unravel the *B120* gene alteration in *B-120* overexpressing neuroblastomas, we speculated the mutated B120 protein accumulated in the cytoplasm and nucleus. Because several exons of the *B120* gene were not amplified from paraffin-embedded tissues, further study using freshly isolated materials might clarify whether and how the *B120* mutation occurred in neuroblastomas. We also examined the sequences of all 17 exons of *B120* (Figure 5) in IMR-32, TGW, and NH-12 neuroblastoma cells; however, no mutation was detected in these cells (data not shown). Ectopic *B120*-transfected IMR-32, TGW, and NH-12 neuroblastoma cells adhered to each other and formed clusters, as described above (Figure 6B). We further identified three genes expressed in the *B120*-transfected neuroblastoma cells (Figures 7 and 8). One of three appeared to be identical to human *p120* gene. The other two genes were novel and designated as *B120ind1* and *B120ind2* (B120-induced gene 1 and 2, respectively). p120 was first identified as proliferation-related nucleolar protein, which is detectable early in the G_1 phase of the cell cycle and peaks early in the S phase.[22] Most human malignant tumors contain much higher levels of protein p120 than normal resting cells.[22] Although the function of p120 is still unclear, yeast Nop2p, which has substantial homology with human p120, is a nucleolar protein that plays an essential role in the maintenance of nucleolar structure.[23] We have been unable to determine the entire
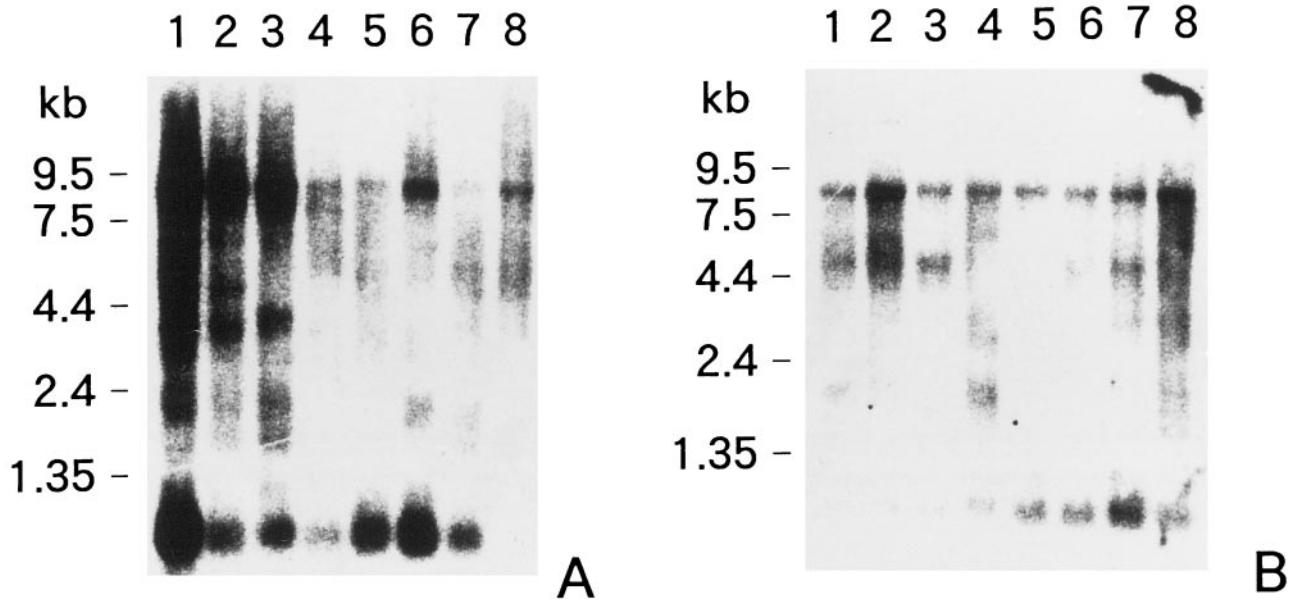
Figure 9. Northern blot analysis of *B120* mRNA in various tissues. **A:** Heart (**lane 1**), brain (**lane 2**), placenta (**lane 3**), lung (**lane 4**), liver (**lane 5**), skeletal muscle (**lane 6**), kidney (**lane 7**), and pancreas (**lane 8**). **B:** Spleen (**lane 1**), thymus (**lane 2**), prostate (**lane 3**), testis (**lane 4**), ovary (**lane 5**), small intestine (**lane 6**), colon (**lane 7**), and peripheral blood lymphocytes (**lane 8**). Each lane contained ~2 μg of poly A+ RNA. Blots were probed with an ~1-kb cDNA corresponding to the 3′ terminus of the *B120* mRNA.

sequence of the other two genes, *B120ind1* and *B120ind2*. As demonstrated in Figure 8, both *B120ind1* and *B120ind2* were induced in *B120*-transfected neuroblastoma cells. Thus, ectopic B120 protein expression in neuroblastoma cells increased or induced the expression of other molecules including p120 nucleolar proliferation-related antigen and affected the morphology of these neuroblastoma cells. Interestingly, we detected no significant bands indicating down-regulation in *B120*-transfected neuroblastoma cells. Consistent with this observation, the Drosophila osa (also termed eyelid) protein, which has strong homology with p270 and is a component of the Drosophila counterpart of SWI/SNF, seemed to promote the activation of specific genes.[24]

The monoclonal antibody to B120 protein used in this study was previously generated by immunizing mice with the C-terminal portion of B120 molecule. The C-terminus of *B120* cDNA product has amino acids different from those of p270, the human homologue of *SWI1* (*SMARCF1*). We re-examined mRNA expression of *B120* gene in various human organs by Northern blotting, as demonstrated in Figure 9.

In the present study, we used a cDNA probe including the 3′ terminus, which is different from that of p270. Several mRNA products were observed in human brain. The 8.0-kb RNA band observed may correspond to previously identified *p270* mRNA.[1,7] However, we observed the additional bands in brain, placenta, and lymphoid tissues. *B120* cDNA contained ~4.5 kb and may be responsible for the 5-kb band. We also detected a 5-kbp band in human spleen, thymus, and peripheral lymphocytes (Figure 9B). As demonstrated in Figure 3, a single band of 120 kd was detected in both original and *B120*-transfected neuroblastoma cells. Notably, the extraction buffer used in this study, 0.5% Nonidet P-40, did not

solubilize cell nuclei. Therefore, it may not be surprising that we detected no significant band in the fetal developing brain. As demonstrated in Figure 1, A and B, immunohistochemical staining with anti-B120 antibody was restricted to the nucleus of subventricular neuroprogenitor cells. Based on the above findings, we concluded that B120 is a natural truncated form of p270, the human homologue of SWI1 (SMARCF1). Very recently, we constructed a pEGFP (eukaryotic green fluorescent protein)-*B120* vector to synthesize recombinant EGFP-*B120* protein and found that fluorescent signal was not always confined to the nucleus but could also be found in the cytoplasm of several tumor cell lines (data not shown).

Our findings together with previous data suggested the hypothesis that the putative mutation of *B120* gene in neuroblastomas, which represented LOH in the *B120* gene region, might contribute to the long turnover of B120 gene product. It is also likely that aberrant B120 expression may induce expression of other proliferation-related genes in neuroblastoma cells. Because we found no mutation in the 17 exons of *B120* in IMR-32, TGW, and NH-12 neuroblastoma cells, *B120* may be not directly linked to initial tumorigenesis of neuroblastomas. However, it is possible that neuroblastomas with genomic instability undergo an unbalanced loss of *B120* gene during progression. Interestingly, a recent completed retrospective study of uniformly treated Children's Cancer Group patients indicated that 1p LOH may identify patients with low- or intermediate-risk features who are more likely to suffer disease relapse.[25] Investigation of a large number of cases is needed to determine whether and how *B120* is involved in the tumorigenesis or biological behavior of neuroblastomas. The promoter region of *B120* gene should also be determined. (Primer sets to

amplify each of the 17 exons including intron-borderline are now available from us by direct request.)

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