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Establishment and Characterization of Choroid Plexus Carcinoma Cell Lines: Connection between Choroid Plexus and Immune Systems

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Murine choroid plexus cell lines were produced from choroid plexus carcinoma generated in transgenic mice harboring the viral oncogene simian virus 40 large tumor antigen under transcriptional control of an intronic enhancer region from the human immunoglobulin heavy chain (IgH) gene. Two morphologically distinct cell lines have been cloned. These established cell lines retained the characteristics of choroid plexus cells in that they expressed such choroid plexus cell marker or related proteins as transthyretin and α_2-macroglobulin. They were tumorigenic in nude mice. In the cell lines, the μA and μB (HE2) motifs within the IgH intronic enhancer were active and we also demonstrated the existence of the proteins binding to these motifs, suggesting a potential link between the choroid plexus and immune systems. It is considered that these binding proteins act as transactivators for the enhancer and may belong to the class of ETS-related proteins. These cell lines and xenografts should be useful materials for analyses of choroid plexus functions.

Key words: Choroid plexus carcinoma — Cell line — Trans-acting factor — Immunoglobulin

Choroid plexus tumors developed in transgenic mice harboring the simian virus 40 (SV40) large tumor antigen (T-Ag) gene have been reported from several laboratories[1–3] and it was demonstrated that the oncogene T-Ag is tumorigenic in choroid plexus. We also previously produced transgenic mice carrying the SV40 T-Ag gene under the transcriptional control of regulatory sequences derived from the human immunoglobulin heavy chain (IgH) intronic enhancer region in order to analyze the function of *cis*-acting DNA motifs within the enhancer *in vivo*. In our transgenic mice, T-Ag was expressed in choroid plexus cells and choroid plexus carcinoma (CPC) developed at high frequency, as well as B-lymphoma. Furthermore, using a deletion mutant of the IgH enhancer, we demonstrated that the μA and μB (HE2) motifs in the IgH enhancer were transcriptionally active in choroid plexus cells.[4] It is known that the μA and μB motifs in the IgH enhancer together play a most important role as *cis*-elements for B-lymphocyte-specific expression and that the binding factors for them are ETS-related proteins (ETS-1 and PU.1).[5–7] These findings suggest the possibility that *trans*-acting factors for these motifs may also be expressed in choroid plexus cells.

Here we describe the establishment and characterization of CPC cell lines derived from the IgH/T-Ag transgenic mice. These cell lines retained the characteristics of native choroid plexus cells. In addition, we demonstrated that the μA and μB motifs act as an enhancer element and that proteins binding to these motifs are present in the CPC cells. We discuss the functions of the choroid plexus, including the possibility of a relationship between the choroid plexus and the immune systems.

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MATERIALS AND METHODS

IgH/T-Ag transgenic mice The origins and characteristics of the IgH/T-Ag transgenic mice have been described in detail.[4] The transgenes contain full-length IgH enhancer or its deletion mutant fragment containing the μA and μB motifs fused to SV40 T-Ag gene.

Establishment of CPC (ECPC) cell lines Choroid plexus tumors were resected from the transgenic mice. The excised tumors were minced, digested with collagenase, washed with medium, and plated in tissue culture flasks in Roswell Park Memorial Institute (RPMI) 1640 medium/2 m*M* L-glutamine/10% fetal calf serum supplemented with penicillin G (100 U/ml) and streptomycin (100 μg/ml). The resultant cultured cells showed active growth *in vitro* from the first and the proliferating cells were designated ECPC. At passage 2, the ECPC cells were subcloned by the limiting dilution method using culture plates of 96 wells. Four clonal cell lines, ECPC-1, -2, -3, and -4, were successfully established. ECPC-1, -2, and -3 showed the same morphology, but ECPC-4 was distinct from the others. In this study, we used the ECPC-3 and -4. These cell lines continue to proliferate *in vitro* for over a year and were stable over many passages.

Transplantation of cell lines into nude mice The CPC cells were inoculated subcutaneously into the flank of homozygous BALB/c *nu/nu* mice (Charles River Japan, Yokohama), 6 to 8 weeks old, at a concentration of 1×10^7 cells per 0.2–0.4 ml of RPMI 1640 medium. The nude mice were monitored for visible and palpable tumors.

Immunofluorescence Histological marker proteins were examined by immunofluorescence using the following antisera; rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (1:100; provided by Dr. J. E. Goldman),[8] anti-transthyretin (TTR) antiserum (1:100; Dako, Copenhagen, Denmark)[9] and anti-α_2-macroglobulin antiserum (1:100; Dako). Cells were plated into 25 cm^2 culture wells containing poly-L-lysine-coated coverslips, and were cultivated for 2 days. The cells on the coverslips were rinsed in phosphate-buffered saline (PBS), fixed with 10% buffered formalin for 30 min, rinsed in PBS and treated with 1% NP-40 in PBS for 10 min. They were then washed with PBS, and incubated sequentially in a diluent (10% goat serum in PBS) for 15 min; primary antibody at 4°C overnight; PBS for 3 min, three times; and FITC-labeled donkey anti-rabbit IgG (1:50 in PBS, Amersham, Buckinghamshire, UK).

Histopathology of xenografts Subcutaneous tumors in nude mice were resected and fixed with 4% paraformaldehyde in a 0.1 *M* phosphate buffer (pH 7.4) for one day or more, and embedded in paraffin. Sections 5 μm thick were deparaffinized and stained with hematoxylin and eosin.

Immunocytochemical staining was carried out using the labeled biotin-streptavidin method.[10] The following antisera were used: anti-GFAP antiserum (1:1000), anti-TTR antiserum (1:500) and anti-S-100 antiserum (1:500; Dako). The sections were deparaffinized in xylene, dehydrated in ethanol and incubated with 0.3% hydrogen peroxide in 100% methanol for 30 min at room temperature to inhibit endogenous peroxidase. They were washed with Tris buffer (50 m*M* Tris-HCl, pH 7.6), then incubated sequentially in 5% non-fat milk with TBS-Tween (50 m*M* Tris-HCl, pH 7.6, 0.5 *M* NaCl, 0.05% NaN_3, and 0.05% Tween 20) for 30 min; with primary antiserum at 4°C overnight; and finally with species-specific biotinylated secondary antibodies and streptavidin-horseradish peroxidase as described by the manufacturer (Amersham). The colored reaction product was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) reaction solution (0.02% DAB/0.003% H_2O_2/50 m*M* Tris-HCl, pH 7.6). The sections were counterstained lightly with hematoxylin.

RNA extraction and Northern blot analysis Total RNA was isolated from the cultured cell lines by the guanidine thiocyanate method and analyzed on a Northern blot. RNA samples (10 μg each) were electrophoresed in 0.75% agarose-6% formaldehyde gels and then transferred to nylon membranes. Probes for Ets-1 and PU.1 were labeled with [α-^{32}P]dCTP using oligo-primed labeling.

DNA transfection into cultured cells and luciferase assay The chloramphenicol acetyltransferase (CAT) gene was separated from the pconaCAT plasmid[11] by digesting the plasmid with *Hin*d III and *Eco*R I and then the luciferase gene was inserted into the same site (pcona-luciferase).[12] The human whole IgH enhancer and its deletion mutant *Mln* I-*Bgl* II fragment, which contains the μA and μB motifs, were cloned into the *Bam*H I site of pcona-luciferase (pEcona-luciferase and pABcona-luciferase).[13]

Cultured cells were incubated at 37°C under 5% CO_2 in air until they were 80% confluent. Aliquots of 1×10^6 cultured cells were then plated in 100-mm tissue culture dishes with 5ml of RPMI-1640 serum-free medium. Plasmid DNA (20 μg) was transfected into these cultured cells by using the Lipofectin™ reagent (BRL, Gaithersburg, MD) following the protocol of the manufacturer. Transfection efficiency for each cell line was evaluated by β-gal assay using a control gene which contains the β-galactosidase gene driven by RSV-LTR. Forty-eight hours after transfection, the luciferase activity of these transfected cells was measured as described.[12]

Nuclear extracts and gel retardation assay Nuclear extracts were prepared by the reported method.[14] All buffers included 5 μg/ml leupeptin, 0.5 m*M* phenylmethylsulfonyl fluoride, 1 m*M* DTT and 2 μg/ml aprotinin. Binding analyses *in vitro* of the nuclear extracts to DNA motifs were examined. DNA probes were synthesized (Biologica, Nagoya) and radioactively end-labeled with [γ-^{32}P]dATP. The same synthesized DNAs were also used for competition assay. The reaction mixtures containing the DNA probes, the nuclear extracts and poly-(dI-dC) (250 ng) in buffer (50 m*M* Tris-HCl pH 7.4, 100 m*M* KCl, 1 m*M* DTT, 10% glycerol) in a final volume of 25 μl were incubated at room temperature for 30 min, in the presence or absence of competitor oligonucleotides. The complexes were electrophoresed on 6% polyacrylamide gel with buffer; 25 m*M* Tris-HCl (pH 8.5), 25 m*M* boric acid and 5 m*M* EDTA. Electrophoresed gels were dried and the retarded protein/DNA complexes were visualized by autoradiography. Oligonucleotides used for probes were as follows:

(i) 2 copies of the μA oligonucleotide
5′-TCGAGGCAGGAAGCAGGGCAGGAAGC-AG-3′
3′-CCGTCCTTCGTCCCGTCCTTCGTCAGC-T-5′

(ii) 2 copies of the μB oligonucleotide
5′-TCGAGTTTAGGAAGCAATTTAGGAAGC-AAG-3′
3′-CAAATCCTTCGTTAAATCCTTCGTTCA-GCT-5′

RESULTS

Production of cultured choroid plexus carcinoma cell lines CPCs occurred frequently in transgenic mice harboring the IgH/T-Ag hybrid gene.[4] From the CPC tissue, murine CPC cell lines were successfully established (see "Materials and Methods"). The cell lines have been designated ECPC followed by an identification number. The cell lines described in this study represent a mixed cell population (ECPC) and two clonal morphologically different cell lines (ECPC-3 and ECPC-4), which have been maintained for over a year and are considered immortal. Population doubling times of 7–9 h were determined for ECPC-3 (passage 16) and ECPC-4 (passage 14) cell lines. The chromosome numbers of metaphase cells revealed the modal peak to be 40 for primary cultured cells (ECPC, passage 5), 80 for ECPC-3 (passage 16), and 57 for ECPC-4 (passage 14). The passage numbers of the cell lines which we used for all other analyses in this work were as follows: passage 5 for ECPC, passages 15–17 for ECPC-3, and passages 13–15 for ECPC-4.

Morphology and phenotypic profile of ECPC cells Both ECPC-3 and ECPC-4 were derived from the same brain tumor. However, their morphology appeared to be different. ECPC-3 cells had elongated cytoplasmic processes and their spindle morphology became prominent at high cell density (Fig. 1A). In contrast, ECPC-4 showed a flattened, polygonal appearance (Fig. 1B). Despite their distinct morphology, both ECPC-3 and ECPC-4 cells were immunopositive with anti-TTR and anti-α_2-macroglobulin, which are authentic markers for choroid plexus cells. Immunofluorescence for TTR showed diffuse, cytoplasmic staining (Fig. 1C) and immunofluorescence for α_2-macroglobulin revealed dot-like localization in the cytoplasm (Fig. 1D). However, these cells were totally

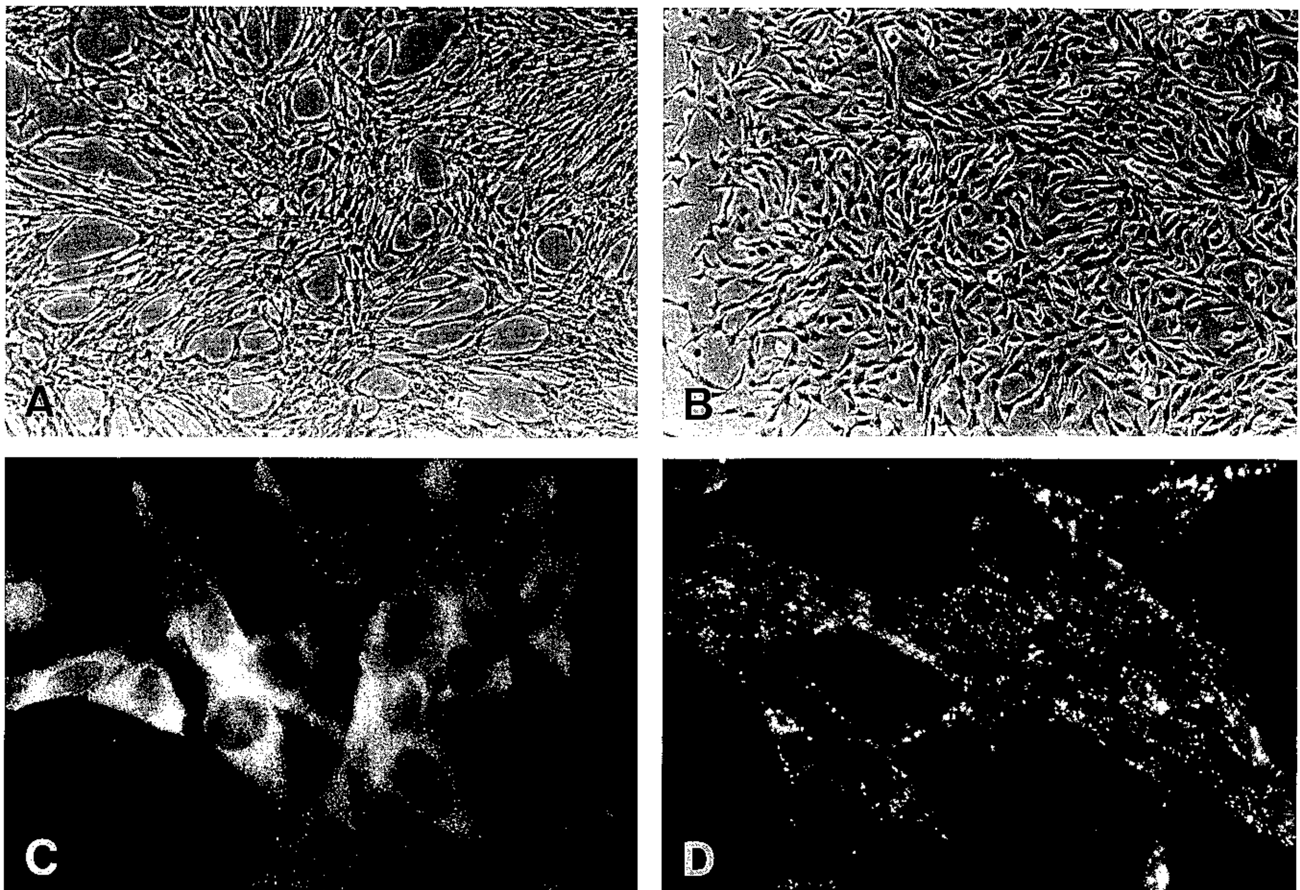

Fig. 1. A and B, Phase-contrast micrographs (original magnification, ×25) of ECPC-3 (passage 17, A) and ECPC-4 (passage 15, B). C and D, Immunofluorescence of transthyretin (C) and α_2-macroglobulin (D) in ECPC-4 (original magnification, ×100).

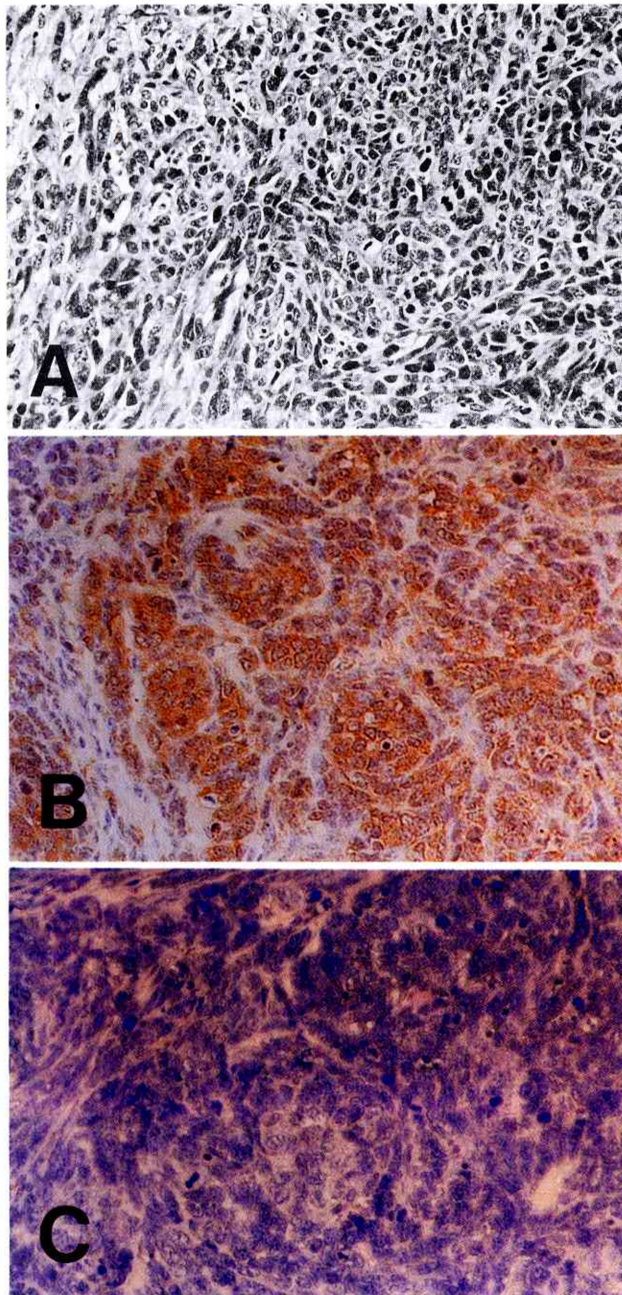

Fig. 2. A, Light micrograph of the xenograft in nude mice (hematoxylin and eosin, original magnification, ×25). B, Immunoperoxidase staining for transthyretin of the xenograft (original magnification, ×25). C, Non-immune serum control of the xenograft (original magnification, ×25).

immunonegative with anti-GFAP which is an astrocytic marker[15] (data not shown). Thus, the nature of choroid plexus cells was retained in these established cell lines.

Transplantation of cultured cell lines into nude mice The primary ECPC cell line was tested for tumorigenicity by subcutaneous inoculations in athymic nude mice. Tumors grew exponentially in nude mice with volume doubling times of 15 days. The predominant cell type was small anaplastic cells arranged in sheets or bundles in high cell density (Fig. 2A). The tumor cells had an oval or elongated hyperchromatic nucleus with occasional bizarre mitotic figures. Immunohistochemically, TTR was expressed in tumor cells as in the CPC cell lines. In particular, tumor cells forming vague clusters in a lobulated fashion tended to show stronger immunoreaction for TTR (Fig. 2B, C). The tumor cells were also immunopositive to α_2-macroglobulin and S-100 protein, but negative to GFAP (data not shown).

Enhancer activity of μA and μB motifs in CPC cells To determine whether the μA and μB motifs within the IgH enhancer (Fig. 3A) are the crucial enhancer element in the CPC cells, we examined the functional activity of these motifs by using the luciferase assay. We constructed three plasmids; pcona-luciferase which has no enhancer fragment, pEcona-luciferase which contains the whole IgH enhancer and pABcona-luciferase which contains the μA and μB motifs (see "Materials and Methods"). Each construct has the conalbumin promoter/luciferase gene in common. We transfected these plasmids into a B-cell line (X63), HeLa cells and established ECPC cells. Then, luciferase activity was measured (Table I). The pcona-luciferase did not produce any luciferase activity in any of the cell lines tested. The whole IgH enhancer showed significant enhancer activity in the B-cell line X63 cells and ECPC cells, but little activity in HeLa cells. In the ECPC cells, the μA and μB motifs showed a fair amount of activity (30.4% of the activity in X63).

CPC cell lines contain binding factors to μA and μB motifs We next investigated the presence of binding proteins to the μA and μB in CPC cells. In B-lymphocytes, binding factors, Ets-1 for μA and PU.1 for μB, were reported.[6] Both binding factors were expressed in B-lymphoma cells derived from our transgenic mice, but were not detected in our CPC cell lines in Northern blot analysis (Fig. 3B) or by the reverse transcription-polymerase chain reaction (RT-PCR) method (data not shown). To determine the existence of proteins that interact specifically with the μA and μB motifs in CPC cells, we performed gel retardation assay with μA and μB oligonucleotides as probes. We compared the abilities of nuclear extracts from different cell types to form complexes with these motifs.

In the assay for μA (Fig. 4A), a similar electrophoretic pattern was observed in B-lymphocytes (WEHI 231) and CPC cells. We could detect two major complexes (complexes 1 and 2) in both cells, though the predominant

Table I. Luciferase Activity of Each Plasmid

Cell line	Transfected plasmid		
	pcona-luciferase activity	pEcona-luciferase activity (%)[a]	pABcona-lusiferase activity (%)[a]
X63	0	3.10 (100)	1.02 (100)
HeLa	0	0.10 (3.2)	0 (0)
ECPC	0	0.95 (30.7)	0.31 (30.4)

a) Percent, relative to the activity in X63 cells.

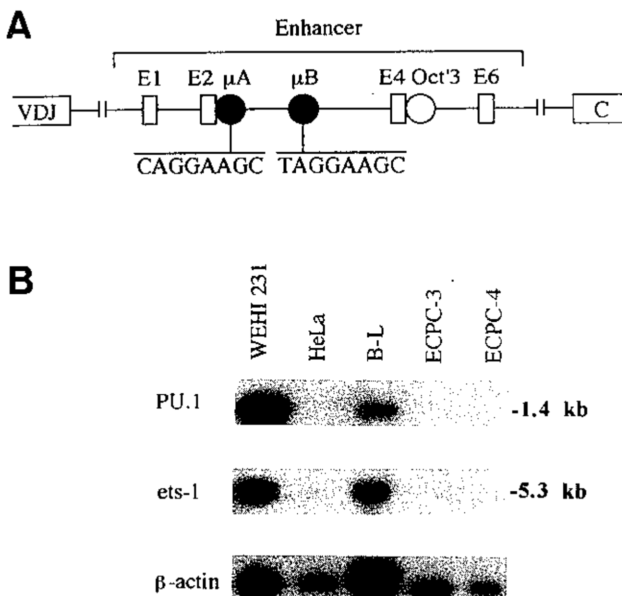

Fig. 3. A, Schema of IgH intronic enhancer. E1, E2, μA, μB, E4, Oct'3, and E6 represent the motifs in the enhancer. VDJ, variable region; C, constant region. B, Northern blot analysis for detection of the expression of the transcripts encoding PU.1 and Ets-1. Cell lines are, from left to right, WEHI 231 (mature B cell), HeLa, B-L (T-Ag positive B-lymphoma cell line derived from our transgenic mouse), ECPC-3, and ECPC-4.

band was complex 1 in CPC cells and complex 2 in B-lymphocytes. In a hepatoblastoma cell line (HepG2), in which IgH enhancer is inactive, a different band (complex 3) was seen. In experiments with the μB probe, a band (complex 4) with slower mobility was found using WEHI 231 cell extracts compared to the complex 5 found in assays with CPC cell extracts (Fig. 4B). No specific band was detected in HepG2 cells. These complex bands disappeared in the presence of a 10-fold excess of competitors (the same oligonucleotides as the probes).

DISCUSSION

In our transgenic mice, the deleted IgH enhancer fragment, which is composed of the μA and μB motifs, was active in choroid plexus cells and was involved in the occurrence of CPCs. The tumor cells were composed of polygonal epithelial cells with high mitotic activity, proliferating in a papillary fashion.[4] Now, we have established CPC cell lines from murine CPC tissue. Two morphologically distinct cell lines were cloned (ECPC-3, -4). Both cell lines retained functional characteristics of choroid plexus cells in that they were immunopositive for such choroid plexus-marker proteins as TTR and α_2-macroglobulin (Fig. 1). The cell lines could be easily transplanted in nude mice. In the xenografts, tumor cells retained the same immunohistochemical characteristics as the cell lines. Chromosome analyses of ECPC-3 and ECPC-4 cell lines indicated that they were murine tumor cell lines with many abnormal chromosomes.

In these cell lines, the μA and μB motifs were transcriptionally active, as shown in luciferase assay. This indicated that the CPC cells contain trans-activators that specifically interact with the μA and μB motifs. Actually, μA- and μB-specific binding proteins were detected in these cells, as shown in gel retardation assays (Fig. 4). Based on the migration pattern in the assay, the predominant complex with μA oligomer or μB oligomer of CPC cells was different from that of B-lymphocytes. It was reported that μA and μB were crucial components of the IgH enhancer for B-lymphocyte-specific activity, and that the *trans*-acting binding factors for them in B-lymphocytes were Ets-1 and PU.1, respectively.[6,7] The μA and μB motifs are composed of purine-rich sequences including the GGAA consensus tetramer for Ets-related proteins[16] and Ets-1 and PU.1 also belong to this class. PU.1 mRNA expression was restricted to lymphoid and macrophage lineages.[17] Ets-1 has been found to be consistently expressed both in cells of the lymphoid lineages[6] and in a subgroup of neuroblastoma.[18] Ets-1 protein has been localized in human astrocytes in brain sections.[19] PU.1 and Ets-1 regulate the expression of many immune-associated molecules.[20–22] However, CPC cell lines ex-

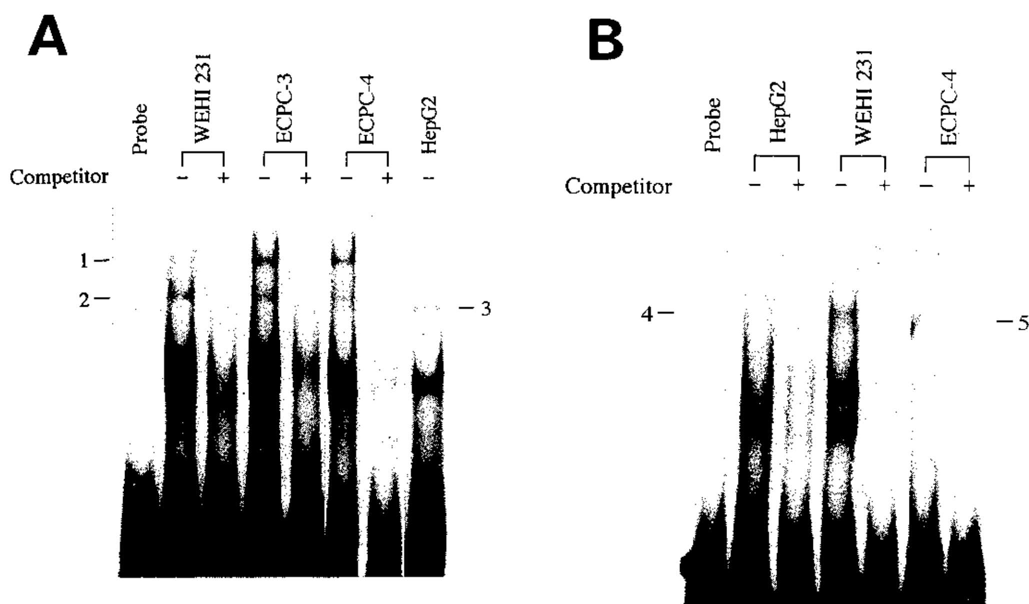

Fig. 4. Nuclear factors interact with the μA (A) and μB (B) sites. Gel retardation assay performed with synthetic oligonucleotides in the presence (+) or absence (−) of competitor oligonucleotides (100 μg). Cell lines, WEHI 231, ECPC-3, ECPC-4 and HepG2 (hepatoblastoma cell line), are indicated above the lanes. The numbers indicate the different DNA-protein complexes.

pressed little or no Ets-1 and PU.1 transcripts (Fig. 3). These findings suggest that other Ets-related proteins in the CPC cells bind to the μA and μB motifs and then activate them, and that these binding proteins also promote the expression of some immune-associated molecules.

It is known that the choroid plexus synthesizes the bulk of cerebrospinal fluid (CSF) and that the CSF is important in regulating the composition of the extracellular environment of the central nervous system. But few details are known about the developmental and physiological roles of the choroid plexus. Nathanson and Chun showed that the choroid plexus has functions relating to immunological reactions by demonstrating that the choroid plexus was able to present foreign antigens to peripheral helper T cells.[23] Sacchi *et al.* suggested that identification of the genes regulated by the Ets-1 family in lymphoid and neural cells would be helpful in elucidating some of the connections between the nervous and immune systems.[18] Our data also support the possibility of a relation between the choroid plexus and the immune systems.

The functions of choroid plexus relating to neural/glial development[24, 25] and neuroendocrinal effect[25, 26] have been examined by means of molecular techniques. Prior to birth, the choroid plexus is much larger in proportion to the rest of brain than in the postnatal period.[25] The choroid plexus may play particularly important roles in fetal development, and may produce and secrete factors required for neural/glial development, growth and proliferation. We observed rapid proliferation of cultivated neuroblastoma cells in serum-free medium supplemented with the conditioned medium of our CPC cells, whereas the cells hardly proliferated in a serum-free condition without the conditioned medium (unpublished observations). Further analysis of cytokines associated with the immunological function of choroid plexus cells will be of interest. Thus, we believe that our established permanent cell lines and xenografts will be useful materials for research on the choroid plexus tissue.

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