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EXPRESSION OF THE RAG-2 GENE IN MURINE CENTRAL NERVOUS SYSTEM TUMOR CELL LINES

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Two tightly linked recombination activating genes, RAG-1 and RAG-2, are involved in VDJ recombination of the immune system. Although these genes were originally thought to be expressed exclusively in precursor B and T cells, RAG-1 transcripts were recently found in the murine central nervous system(CNS)[Chun et al., Cell 6, 189, (1991)]. We found that the RAG-2 gene was expressed in CNS tumor cell lines, melanoma(B-16) and skin fibroblast(A9). RAG-1 expression was not found in any non-lymphoid cell lines examined including CNS tumor cell lines. Another VDJ recombination-related gene RBP-Jκ was expressed in all tumor cell lines examined. It remains to be seen whether expression of RAG-1 and RAG-2 in CNS is abortive or functionally important.

Recombination plays an important role in the life cycle of a variety of organisms, in which it is used to mediate genetic mobility and gene assembly, and to regulate gene expression. Studies on conjugational recombination in *Escherichia coli* have been pivotal in our understanding of bacterial genetics. But, the molecular mechanism of recombination following conjugation has remained obscure(1). In vertebrae, somatic DNA recombination is required for the activation and diversification of antigen receptor genes in lymphocytes(2). The variable region genes for both the immunoglobulin and T cell receptor are split into variable(V), diversity(D), and joining(J) segments on the germ-line chromosome. During lymphocyte differentiation the gene segments are joined together by site-specific DNA recombination events, known as VDJ joining. VDJ recombination of the immunoglobulin and T cell receptor genes requires conserved recombination signal sequences(RS) of a heptamer CACTGTG and a T-rich nonamer GGTTTTTGT. There are at least 4 gene products that seem to be involved in VDJ recombination. First, a specific binding protein (RBP-Jκ) to JκRS has been isolated and its cDNA has been characterized (3, 4). Secondly, *scid* mutation has been shown to cause inaccurate and inefficient VDJ recombination in both B and T lymphocytes(5). Finally, VDJ recombination is activated by the

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expression of two tightly linked recombination activating genes, RAG-1 and RAG-2 (6, 7).

Originally, RAG-1 and RAG-2 expression was found only in precursor B and T cells of mammalian tissues (8). But recently, the mouse central nervous system (CNS) was shown to contain low levels of the RAG-1 transcript, but not the RAG-2 transcript (9). Similarities between the CNS and the immune system have been suggested by sharing some of polypeptide signaling molecules, transcription factors, cell surface antigens and immunoglobulin supergene family members (10, 11, 12). It is appealing that the CNS utilizes site-specific recombination similar to that in the immune system. However, transgenic mice containing the exogenous interleukin 2 receptor gene flanked by RS showed no sign of DNA rearrangement in CNS (13).

To study the physiological significance of VDJ recombination-related gene expression in CNS, we have checked the expression of the RAG-1, RAG-2 and RBP-Jκ genes in several mouse CNS and non-CNS tumor cell lines. We report here that all CNS tumors examined express the RAG-2 gene but not the RAG-1 gene.

MATERIALS AND METHODS

Tumor cell lines. All tumor cell lines are derived from mouse. A methylcholantherene-induced malignant glioma cell line(ependymoblastoma, 203-glioma)(14), a Schmitt-Ruppin Rous sarcoma virus-induced glioma(RSVM)(15, 16), a spontaneous glioma(GM-glioma)(15, 16), a spontaneous neuroblastoma(C-1300)(17), a spontaneous melanoma(B-16)(18), an androgen-responsive mammary cancer(SC115)(19), an ultraviolet induced fibrosarcoma (UV ♀ 1)(20), a methylcolantherene-induced fibrosarcoma(YM-12)(15, 16), a pre-B cell line(38B9)(21), and a multipotential stem cell line(LyD9)(22) were subcloned and maintained by *in vitro* passage in our laboratory. A mammary cancer(BALB-MC, E12), a mammary carcinoma(FM3A), a spontaneous mammary tumor(MMT060562), Ehlich ascites(Ehrlich-lettre ascites carcinoma), three teratocarcinoma cell lines(F9-49, OTT6050, F9), a hepatoma(MH134), a liver cell line(NCTC clone 1469), a Leydig cell testicular tumor(I-10), an estrogen-dependent Leydig cell tumor(B-1), an adrenal tumor(Y-1), a connective tissue clone of strain L(NCTC clone 929), a skin fibroblast (A9), a sarcoma 180(CCRF S180II), a contact inhibited embryo(BALB/3T3, clone A31), and an SV virus-transformed BALB/3T3 embryo(SV-T2) were kindly supplied by Japanese Cancer Research Resource Bank(JCRB). These cells were cultured with Dulbecco's modified MEM or RPMI 1640(Nissui, Japan) with 10 % heat inactivated fetal calf serum (GIBCO, Grand Island, NY), 2mM glutamine, 50μM 2-mercaptoethanol, and antibiotics.

RNA PCR reaction assay. Total cytoplasmic RNA was prepared from cell lines as the standard NP-40 lysis method(23). cDNA was synthesized using total RNA sample as template. Total RNA(10μg) was incubated at 42°C for 60 min with a mixture of 100 units of reverse transcriptase(Bethesda Research Laboratories, Gaithersburg, USA),50mM Tris-HCl(pH8.3), 75mM KCl,10mM DTT, 3mM $MgCl_2$, 10 mM each dNTP, 20 units of RHase inhibitor(Promega, Madison, USA) and 0.2 μg of DNA random hexamers(Takara Shuzo Co., Kyoto, Japan) in a

volume of 30 μl. Thereafter the reaction mixture was incubated at 65°C for 5 min. An aliquot(10μl) of reverse transcribed products was mixed with 2 units of *Taq* DNA polymerase(Takara Shuzo Co., Kyoto, Japan), and 0.5 μg each of sense and antisense primers in a buffer containing 20 mM Tris-HCl(pH8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 100μg/ml BSA and 10 mM each dNTP in 50 μl. The mixture was overlaid with 30-μl mineral oil to prevent evaporation and then amplified by 50 cycles of PCR(Perkin-Elmer, Cetus). A thermal cycle profile used in this study is; a) denaturing for 30 sec at 95°C, b) annealing primers for 60 sec at 55°C, and c) extending the primers for 60 sec at 72°C. A portion(10μl) of the PCR mixture was electrophoresed in a 2% agarose gel in 0.04M Tris-acetate and 0.001M EDTA buffer. The gel was stained with ethidium bromide and photographed. The oligonucleotides used in these experiments were as follows:

RAG-1;	(10→29),	5′-CTGTGGCATCGAGTGTTAAC-3′	(sense strand)
	(668→649),	5′-TGACTGTGGGAACTGCTGAA-3′	(anti-sense strand)
RAG-2;	(76→95),	5′-TAAGAAAGAGTATTTCACAT-3′	(sense strand)
	(582→563),	5′-TCAATGGAATGGCCGTATCT-3′	(anti-sense strand)
RBP-Jκ;	(752→771),	5'-TGGACGACGACGAGTCGGAA-3'	(sense strand)
	(985→966),	5'-CTTGAGAAAGGCACAAGTAC-3'	(anti-sense strand)

Southern blotting of PCR products. A 10-μl aliquot of each PCR reaction was electrophoresed through a 2.0% agarose gel and then transferred to a nylon membrane in 1.5M NaCl and 0.15M sodium citrate(10xSSC) overnight. Southern blot hybridization was carried out using the standard method(12). The membrane was exposed to an X-ray film backed with an intensifying screen at -70°C.

Northern blot analysis. poly(A)$^+$ mRNA was prepared from total RNA using oligo(dT)-latex(Takara Shuzo Co., Kyoto, Japan) as described(23). RNA samples were electrophoresed in 1% agarose gels containing 2.2M formaldehyde and transferred to a nylon membrane. Hybridization with probe was carried out in 50% formamide, 5xSSC, 50mM sodium phosphate(pH6.5~6.9), 2 mg/ml salmon sperm DNA, 10xDenhardt's at 42°C overnight. Final washes were with 0.1xSSC-1.0% SDS at 42°C. The membrane was exposed to an imaging plate and analyzed by Image Analyzer(Fuji Film, Tokyo, Japan).

Probes. Probes were labeled with [α-32P]dCTP using hexamer labeling method (23). Probes used in these experiments were as followed: RAG-1, *Bgl* I-*Bam*H I fragment from PUC19-RAG-1(6); RAG-II, *Pst* I-*Eco*R V fragment from pBM030-RAG-II (7); β-actin, *Pst* I fragment from pAL41-β-actin (24)

RESULTS AND DISCUSSION

To screen a variety of tumor cell lines, we used a PCR assay for specific detection of RAG-1, RAG-2 and RBP-Jκ gene transcripts(Table 1). All tumor cell lines examined were negative for RAG-1 gene transcripts except for 38B9 cells(positive control). But, all CNS tumor cell lines examined, C-1300(neuroblastoma), 203-glioma(ependymoblastoma), RSVM(glioma) and GM-glioma, expressed the RAG-2 gene. Two non-CNS tumor lines, B-16(melanoma), and A9(skin fibroblast), were also positive for RAG-2 gene expression. PCR-amplified RAG-2 cDNAs from the CNS tumor cell lines and two others were

similar in size with that from 38B9 cells(positive control), and hybridized with RAG-2 probe(Figures 1 and 2). Relative intensities of PCR-amplified bands of different cell lines varied between experiments probably because of less quantitative nature of the PCR reaction but intensities of the PCR-amplified bands had a good correlation with those of the hybridized bands. All PCR products with RAG-2 primers were digested with two restriction enzymes(*Pst* I and *Bgl* I), and the resulting DNA fragments were exactly as expected from the known sequence of RAG-2 cDNA(data not shown).

Table 1

RAG-1, RAG-2 and RBP-Jκ expresion in tumor cell lines

	Cell name	character	RAG-1	RAG-2	RBP-Jκ
1	203-glioma	methylcolantherene induced ependymoblastoma	–	+	+
2	RSVM	Schmitt-Ruppin Rous sarcoma virus-induced glioma	–	+	+
3	GM-glioma	spontaneous glioma	–	+	+
4	C-1300	spontaneous neuroblastoma	–	+	+
5	B-16	spontaneous melanoma	–	+	+
6	SC115	mammary cancer	–	–	+
7	BALB-MC, E12	mammary cancer	–	–	+
8	FM3A	mammary carcinoma	–	–	+
9	MMT060562	spontaneous mammary tumor	–	–	+
10	Ehrlich ascites	Ehrlich-letter ascites carcinoma	–	–	+
11	F9-41	teratocarcinoma	–	–	+
12	OTT6050	teratocarcinoma	–	–	+
13	F9	teratocarcinoma	–	–	+
14	MH134	hepatoma	–	–	+
15	NCTC clone 1469	liver	–	–	+
16	I-10	Leydig cell testicular tumor	–	–	+
17	B-1	estrogen-dependent Leydig cell tumor	–	–	+
18	Y-1	adrenal tumor	–	–	+
19	UV♀1	ultraviolet induced fibrosarcoma	–	–	+
20	YM-12	methylcolantherene induced fibrosarcoma	–	–	+
21	NCTC clone 929	connective tissue, clone of strain L	–	–	+
22	A9	skin fibroblast, AG-γ	–	+	+
23	CCRF S180 II	sarcoma 180	–	–	+
24	BALB/3T3, clone A31	embryo, contact inhibited	–	–	+
25	SV-T2	SV virus-transformed BALB/3T3 embryo	–	–	+
26	38B9	Pre B cell	+	+	+
27	LyD9	multipotential stem cell line	–	–	+

+, expected size cDNA was amplified by PCR. –, no hybridizable cDNA was amplified by PCR.

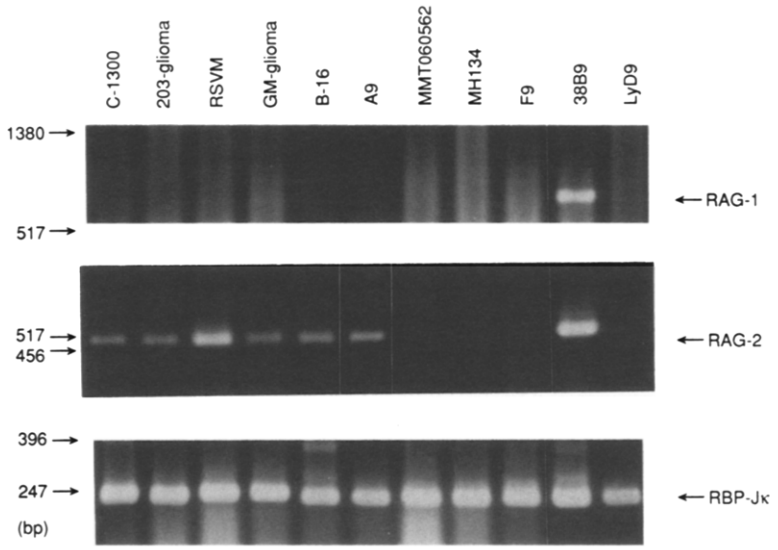

Figure 1. PCR analyses of RAG-1, RAG-2 and RBP-Jκ mRNA in tumor cells. PCR assays were repeated at least three times as described in Materials and Methods. Only representative data are shown. Sizes of amplified DNA are indicated on left. Expected sizes of RAG-1, RAG-2 and RBP-Jκ DNA are indicated on right.

To confirm that the full-length RAG-2 mRNA is expressed in the CNS tumor cell lines and two other lines, we have done Northern blot analysis of poly(A)$^+$ RNA from representative 3 tumor cell lines(C-1300, 203-glioma, and B-16). C-1300, 203-glioma and B-16 RNAs were all positive for 2.1-kb RAG-2 mRNA (Figure 3). When the filter was rehybridized with RAG-1 probe, these tumor cell

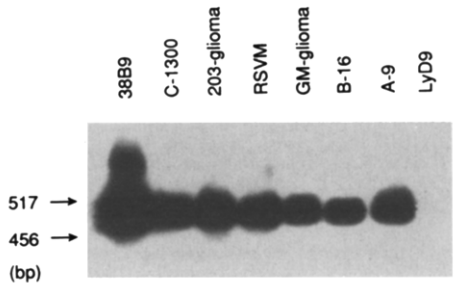

Figure 2. Southern blotting analysis of PCR products with RAG-2 probe. An aliquot (10μl) of each PCR reaction of cell line RNA indicated was electrophoresed through a 2.0% agarose gel and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labeled RAG-2 probe. The PCR reactions were different from those shown in Figure 1.

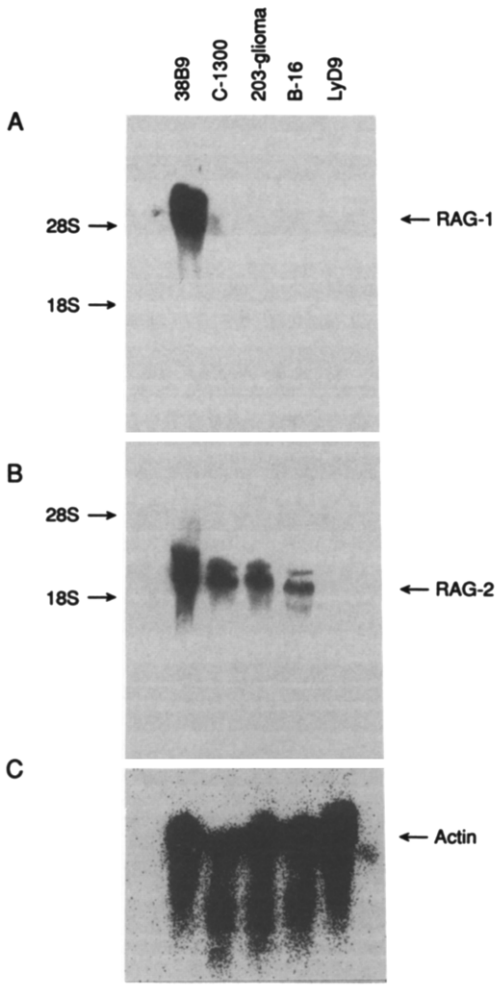

Figure 3. Northern blotting analysis of RAG-1 and RAG-2 mRNA in tumor cell lines. Northern blot filters containing poly(A)$^+$ RNA(20~30μg) from the representative tumor cell lines were hybridized with RAG-1, RAG-2 and β-actin probes and exposed to an imaging plate and analyzed by Image Analyzer.

lines were negative for RAG-1 gene expression(Figure 3). All tumor cell lines were positive in RBP-Jκ gene expression as assessed by PCR and Southern hybridization of the amplified products (Figure 1).

We have demonstrated by PCR and Northern blot analyses that the RAG-2 gene is transcribed in all CNS tumor cell lines examined and two other non-lymphoid cell lines, whereas the RAG-1 gene is not transcribed in any non-lymphoid cell lines examined. The RBP-Jκ gene is transcribed in all tumor cell lines examined. There is no chance of contamination of lymphoid cells because all these cells were cultured *in vitro*. Since no antibodies are available against RAG-1 and RAG-2 proteins, we cannot tell whether RAG-2 protein is actually expressed in CNS tumors.

Several hypotheses may be put forward to explain these data. First, the RAG-2 gene expression may be fortuitous and have no biological function in CNS. Secondly, the RAG-2 gene transcript in CNS tumor cell lines may have an unknown function different from the site-specific recombination that takes place in the immune system. Carson et al. (25) suggested that the RAG-1 and RAG-2 genes may have independent roles in other recombination systems, although both RAG-1 and RAG-2 genes are required for VDJ recombination. Thirdly, the RAG-2 gene may have a role in site-specific recombination in CNS in combination with another protein. Although Chun et al.(9) reported the presence of low levels of RAG-1 transcripts in CNS, we could not detect RAG-1 transcripts in CNS tumors even by the PCR method. On the other hand, the RBP-Jκ gene is expressed in all tumor cell lines examined. This gene may have another important functions as proposed elsewhere(26).

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