The Proto-Oncogene *c-myb* is Expressed in Sporadic Bovine Lymphoma, but Not in Enzootic Bovine Leukosis

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ABSTRACT. We examined bovine *c-myb* gene expression in six samples of sporadic bovine lymphomas (two calf, three thymic and one intermediate) and five of enzootic bovine leukosis. Tumor cells of the sporadic bovine lymphomas were of immature cell lineage (one B lymphoma and five T lymphomas). The *c-myb* mRNA was expressed in almost all the sporadic bovine lymphomas (except for one thymic form) including a BoCD8 single positive T lymphoma. On the contrary, *c-myb* was not expressed in mature B lymphomas of enzootic bovine leukosis. The results suggest that c-*myb* expression is closely associated with tumor cell differentiation of bovine lymphomas.
— KEY WORDS: *c-myb*, enzootic bovine leukosis, phenotype, sporadic bovine lymphoma.

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Bovine leukosis is a malignant tumor of the lymphoid cells. Four epidemiological and anatomical forms have been described: adult multicentric, cutaneous, adolescent thymic (TBL) and multicentric calf (juvenile) (CBL) [5]. The adult multicentric form is enzootic (enzootic bovine leukosis, EBL), whereas the other three forms are sporadic (sporadic bovine lymphoma, SBL). EBL is caused by the bovine leukemia virus (BLV) [14], which principally infects B lymphocytes, although T lymphocytes can also be infected [23]. SBL is unrelated to BLV [18]. Clinico-pathologically, CBL and TBL seem to be two extremes in a single entity of SBL [19] and an intermediate form has been classified.

The *myb* oncogene was first identified as the transforming gene of two retroviruses, avian myeloblastosis virus and E26, both of which cause myeloblastic leukemia in birds [16]. The *c-myb* oncogene is expressed at high levels in hematopoietic cells, where it plays a major role in the regulation of human normal and malignant hemopoiesis *in vitro* [2]. The expression of *c-myb* mRNA on murine erythroleukemic cell lines declines during terminal differentiation [21]. Detectable amounts of *c-myb* are also found in various tumors of embryonic origins such as small cell lung carcinoma [10], colon carcinoma [1], and neuroblastoma [24].

Ishiguro *et al.* [11] have reported that *c-myb* mRNA is specifically expressed in double negative T lymphoid cells derived from CBL and TBL. We found a BoCD8 single positive tumor in TBL [3]. Whether or not *c-myb* is expressed in other lymphomas, remains unknown. To determine the relationship between *c-myb* expression and the various types of bovine lymphomas, we examined *c-myb* gene expression in tumor cells derived from SBL and EBL.

MATERIALS AND METHODS

Animals: Samples were obtained from eleven cattle necropsied at the Department of Veterinary Pathology, Iwate University, between January 1991 to October 1994 (Table 1). They were categorized as SBL and EBL, based on clinical findings, necropsy, and the immunodiffusion test for BLV antigens [19].

Gross and histologic examination: All cattle were euthanized and necropsied immediately. Blocks from all organs and tissues were obtained and fixed in 10% neutral formalin, embedded in paraffin, sectioned at 4 μm in thickness, and stained with hematoxylin and eosin (HE) for histologic examination.

Immunohistologic examination: Tumor samples were cut into slices of about 1 × 1 × 0.5 cm, then frozen rapidly in liquid nitrogen and processed as described [4]. Cryosections were made and immunohistologically stained using monoclonal antibodies (mAbs). The mAbs include: anti-BoCD3, MM1A [20]; anti-BoCD4, IL-A12 [15]; anti-BoCD5, CACT105A [8]; anti-BoCD8, CACT80C [8]; anti-WC1-N2, BAQ4A [8, 20]; anti-B-B2, BAQ44A [15]; anti-sIgM, PIg45A [8]; anti-MHC class II HLA-DR-like, TH14B [8]. All the mAbs were applied to the sections at 15 μg/m*l* in phosphate buffered saline (PBS). Positive reactions were detected by means of the avidin-biotin-peroxidase complex (ABC) method [6] using a commercial kit (Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA). Details of the staining procedure have been reported [3].

Nested double polymerase chain reaction (PCR): We determined whether or not tumor tissues contain BLV proviral sequences, by means of nested double PCR as described [3], using genomic DNA as a template and primers to the pX region of the BLV genome [22]. Chromosomal

Table 1. Clinical and hematologic findings of bovine lymphomas

Specimen No.	Breed[a]	Sex[b]	Age[c]	ID[d] gp, p	BLV provirus[e]	Hematologic findings RBC	Hematologic findings WBC	Clinical diagnosis
SBL 1	JB	M	5m	–, –	–	775	24,500	CBL
SBL 2	JS	M	2m	–, –	–	614	19,400	CBL
SBL 3	HF	F	3y	–, –	–	453	6,400	CBL-TBL
SBL 4	HF	F	4m	–, –	–	566	170,000	TBL
SBL 5	HF	F	2y	–, –	–	421	25,700	TBL
SBL 6	HF	F	2y	–, –	–	174	13,000	TBL
EBL 1	JS	F	8y	+, –	+	555	42,900	EBL
EBL 2	JB	F	8y	+, –	+	543	39,300	EBL
EBL 3	HF	F	4y	+, –	+	400	31,000	EBL
EBL 4	JS	F	9y	+, –	+	508	14,500	EBL
EBL 5	HF	F	3y	+, –	+	200	28,000	EBL

Notes: a) JB, Japanese Black; JS, Japanese Shorthorn; HF, Holstein-Friesian. b) M, Male; F, Female. c) m, months; y, years. d) ID, Immunodiffusion test, gp and p mean serum antibodies to glycoprotein and protein antigen of BLV, respectively. e) BLV proviral DNA was detected by nested double PCR. +, positive, –, negative.

DNA was obtained from frozen tissues sampled for immunohistologic examination, sodium dodecyl sulfate (SDS) and phenol-chloroform extraction.

Preparation of RNA and Northern hybridization: Total cellular RNA was extracted from the tumor tissues of eleven cattle using guanidium isothiocyanate [7]. Poly(A)$^+$ RNA from the tumor tissues was selected on oligo (dT)-cellulose or Oligotex-dT30 (Super) (JSR, Co., Ltd., Tokyo and Roche, Co., Ltd., Tokyo). Extracted cellular RNA (20 μg) or poly (A)$^+$ RNA (2 μg) was denatured with glyoxal and dimethyl sulfoxide, and resolved by 1% agarose gel electrophoresis. The separated RNAs were transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) and hybridized with a radiolabeled DNA probe. Prehybridization and hybridization proceeded in medium containing 50% formamide at 42 °C. The membranes were washed 3 times in 2 × SSC (0.15 M NaCl, 15 mM sodium citrate) and 0.1% SDS for 30 min at 65 °C. The 669 bp *c-myb*-specific DNA fragment [12] and 1.7 kb bovine lactate dehydrogenase-A isozyme (LDH-A) gene [13] were used as probes.

Reverse transcriptase polymerase chain reaction (RT-PCR): RT-PCR was performed using oligo (dT)-primed single-stranded cDNA as a template. The RNA-DNA complex templates were denatured at 94 °C for 5 min, then amplified by 35 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min. The primers were in bovine c-*myb* exon 9 [12] (958-977, 5'- CAGAACCACACATGCAGCT A-3', 1189–1208, 5'-GAATCTATAAATTGAAGTGT- 3').

RESULTS

The clinical diagnoses are described in Table 1. SBL Nos. 1 and 2 were diagnosed as CBL. These animals were affected before they were 6 months old and had multicentric lymphoma in lymph nodes. SBL Nos. 4–6 were diagnosed as TBL, with a tumor mass around cervical regions of the thymus. SBL No. 3 was diagnosed as CBL-TBL intermediate form; the animal had multicentric lymphoma in the lymph nodes but she was 3 years old. The BLV genome was undetectable in all SBL by nested double PCR.

EBL Nos. 1–5 formed a tumor mass mainly in the abdominal cavity (abomasum and mesenteric lymph nodes). The BLV pX-specific band (280 bp) was detected in all EBL specimens.

The tumorous lymphoid cells of SBL No. 2 were slightly small to medium in size (Fig. 1), SBL No. 6 had medium to large lymphoid cells (Fig. 2). The other SBL consisted of medium-sized tumor cells (Fig. 3). The tumor cells of SBL Nos. 1–4 had round or occasionally polymorphic nuclei with a rough chromatic pattern, whereas those of SBL Nos. 5 and 6 had only round nuclei with a similar pattern.

The results of the immunohistologic examination are shown in Table 2. Tumor cells of SBL Nos. 2–3, 5–6 were of the BoCD4 and BoCD8 negative T-cell lineage. In the CBL, tumor cells of SBL No. 1 were positive for molecules expressed on B cells (MHC II, sIgM and B-B2) (Fig. 4), whereas those of SBL No. 2 were positive for BoCD3 (Fig. 5). In the intermediate form, tumor cells were positive for WC1-N2 molecule (Fig. 6) expressed on a major subset of $\gamma\delta$ T-cells in ruminants. In TBL, the tumor cells of SBL No. 4 were positive for BoCD8 (Fig. 7). Tumor cells of SBL No. 5 were positive only for anti BoCD5 (Fig. 8). In SBL No. 6, the tumor cells were weakly positive for BoCD3 and BoCD5.

Tumor cells of all EBL samples were positive for B-cell molecules. EBL Nos. 1–3 contained BoCD5-positive B-cells (MHC II and B-B2). EBL No. 4 consisted of BoCD5-negative B-cells (MHC II and B-B2) as did EBL No. 5, which was also positive for sIgM.

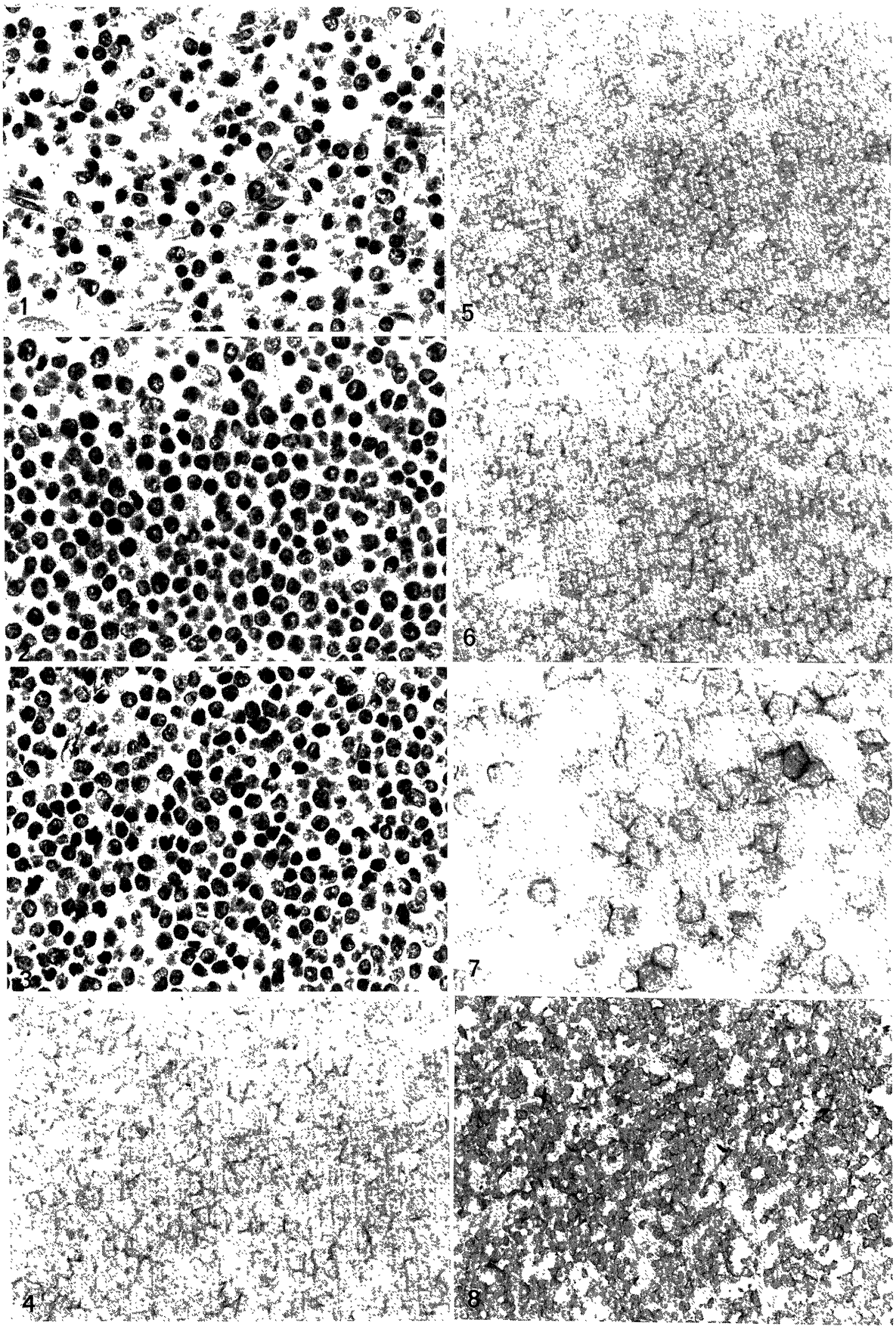

Fig. 1. Superficial cervical lymph node from SBL No. 2. The tumorous lymphoid cells are slightly small to medium in size, with round or polymorphic nuclei and a rough chromatic pattern. HE stain. × 290.

Fig. 2. Neoplastic tissue from the liver capsule of SBL No. 6. The tumorous lymphoid cells are medium to large in size, with round nuclei and a rough chromatic pattern. HE stain. × 290.

Fig. 3. Tumor tissue from SBL No. 5. The tumorous lymphoid cells are medium in size, with round nuclei and a rough chromatic pattern. HE stain. × 290.

Fig. 4. Tumor tissue from the kidney of SBL No. 1. Most tumor cells are positive for MHC class II according to immunohistologic staining using the mAb TH14B. ABC, methyl green counterstain. × 290.

Fig. 5. Superficial cervical lymph node from SBL No. 2. Most tumor cells are positive for BoCD3 according to immunohistologic staining using the mAb MM1A. ABC, methyl green counterstain. × 290.

Fig. 6. Superficial cervical lymph node from SBL No. 3. Most tumor cells are positive for WC1-N2 according to immunohistologic staining using the mAb BAQ4A. ABC, methyl green counterstain. × 290.

Fig. 7. Spleen from SBL No. 4. Most tumor cells are positive for BoCD8 according to immunohistologic staining using the mAb CACT80C. ABC, methyl green counterstain. × 570.

Fig. 8. Superficial cervical lymph node from SBL No. 5. Most tumor cells are positive for BoCD5 according to immunohistologic staining using the mAb CACT105A. ABC, methyl green counterstain. × 290.

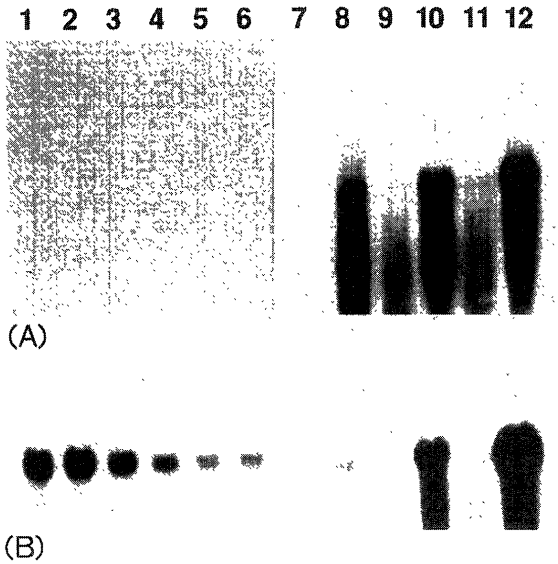

Fig. 9. Northern blots of total RNAs from SBL and EBL. Total cellular RNA (20 μg) from BTL32 and SBL, and 2 μg of poly (A)$^+$ RNA from EBL were separated on a 1% agarose gel, blotted onto a nylon membrane, then hybridized to a bovine *c-myb* probe (A) and rehybridized to an LDH-A specific probe (B). *Lane 1*, EBL No.1; *lane 2*, EBL No. 2; *lane 3*, EBL No. 3; *lane 4*, EBL No. 4; *lane 5*, EBL No. 5; *lane 6*, SBL No. 6; *lane 7*, SBL No. 1; *lane 8*, SBL No. 4; *lane 9*, SBL No. 2; *lane 10*, SBL No. 3; *lane 11*, SBL No. 5; *lane 12*, BTL32.

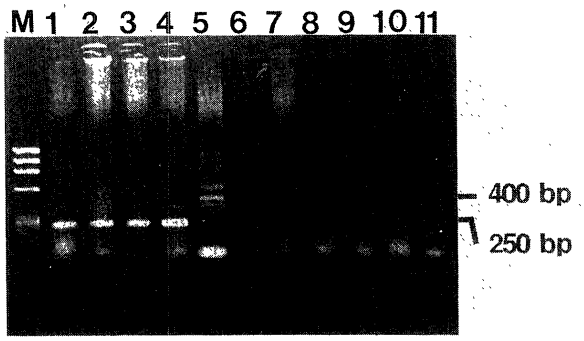

Fig. 10. Detection of the *c-myb* gene by RT-PCR. *Lane 1*, SBL No. 1; *lane 2*, SBL No. 2; *lane 3*, SBL No. 3; *lane 4*, SBL No. 4; *lane 5*, SBL No. 5; *lane 6*, SBL No. 6; *lane 7*, EBL No. 1; *lane 8*, EBL No. 2; *lane 9*, EBL No. 3; *lane 10*, EBL No. 4; *lane 11*, EBL No. 5; M, size markers, øX174/*Hae* III digest.

Northern blots showed that SBL Nos. 2–5 expressed *c-myb* mRNA (about 4.0 kb, Fig. 9A, *lanes 8–11*). In SBL No. 1, the results of Northern blotting were unclear (Fig. 9A, *lane 7*). However, RT-PCR detected a band of *c-myb* mRNA (Fig. 10, *lane 1*). The size of the RT-PCR products was about 250 bp, in agreement with the predicted size of *c-myb* exon 9, in SBL Nos. 1–4 (Fig. 10, *lanes 1–4*), whereas SBL No. 5 generated a larger DNA band (about 400 bp, Fig. 10, *lane 5*). Northern blots and RT-PCR did not detect *c-myb* mRNA in SBL No. 6.

Northern blots of total RNA did not reveal *c-myb* mRNA in any of the EBL samples (data not shown). Therefore, we used mRNA prepared from total RNA of EBL tumor cells for Northern blotting, but no *c-myb* mRNA was detected in any EBL tumors (Fig. 9A, *lanes 1–5*). In EBL No. 5, RT-PCR detected a band of about 250 bp.

In SBL Nos. 1 and 2, LDH-A gene expression was unclear because these were old samples. However, LDH-A transcripts from the other samples were obvious (Fig. 9B).

DISCUSSION

To determine the involvement of *c-myb* expression in the tumorigenicity of bovine lymphomas, we examined *c-myb* expression in SBL and EBL tumors. The *c-myb* transcripts were abundant in the SBL (except for one TBL; SBL No. 6) according to Northern blotting and RT-PCR. However, Northern blotting did not detect *c-myb* mRNA in the EBL, although the *c-myb* message was amplified by RT-PCR from only one EBL sample (EBL No. 5). These results indicate that high, steady levels of *c-myb* mRNA expression are closely associated with SBL, but not with EBL tumors.

A high level of *c-myb* expression has been predominantly detected in immature murine hematopoietic cells, and it is down-regulated during differentiation [9]. Most of the T-cell tumors from SBL that we examined, consisted of double negative (BoCD4$^-$, BoCD8$^-$) T-cells which are immature

Table 2. Immunohistologic findings of neoplastic cells and expression of *c-myb* gene

Specimen No.	Diagnosis	Differentiation antigen								Expression of *c-myb* gene	
		BoCD3	BoCD4	BoCD5	BoCD8	WC1-N2	B-B2	sIgM	MHC II	Northern	RT-PCR
SBL 1	CBL	–	–	NE	–	–	+	+	+	–	+
SBL 2	CBL	+	–	NE	–	–	–	–	–	+	+
SBL 3	CBL-TBL	+	–	+/–	–	+	–	–	–	+	+
SBL 4	TBL	+	–	NE	+/–	–	–	–	–	+	+
SBL 5	TBL	–	–	+	–	–	–	–	–	+	+
SBL 6	TBL	+/–	–	+	–	–	–	–	–	–	–
EBL 1	EBL	–	–	+	–	–	+	–	+	–	–
EBL 2	EBL	–	–	+	–	–	+	–	+	–	–
EBL 3	EBL	–	–	+	–	–	+	–	+	–	–
EBL 4	EBL	–	–	–	–	–	+	–	+	–	–
EBL 5	EBL	–	–	–	–	–	+	+	+	–	+

Notes: –, negative; +/–, weakly positive; +, positive. NE, Not examined.

in terms of T-cell ontogeny. Although tumor cells from SBL No. 4 were positive for BoCD8, the surface expression intensity was lower compared with that of mature T-cells in the peripheral blood determined by flow cytometry (data not shown). Therefore, BoCD8-weakly positive T-cells in SBL No. 4 may be immature BoCD4$^-$ and BoCD8^{low} T-cells prior to becoming double positive T-cells.

High levels of *c-myb* transcripts have been correlated with the pre-B cell stage of development in murine B lymphoid tumor [4]. The tumor cells from EBL in this study did not express the *c-myb* message, because they were mature B-cells. BLV-infected cattle develop lymphoma after long latency periods. It is not clear whether or not the immature B-cells infected with BLV differentiate to mature B-cells under the influence of some soluble factors from T-cells and persistent stimulation of BLV antigen, or whether the mature B-cells are targets of BLV and subsequently proliferate, resulting in persistent lymphocytosis. Therefore, it is necessary to determine the developmental stage of tumor cells of EBL No. 5 (*c-myb* positive by RT-PCR) by rearrangement analysis of the immunoglobulin gene.

The *c-myb* exon 9 contains a leucine zipper structure in the negative regulatory domain. A c-Myb mutant containing an impaired leucine zipper has been described, which does not show negative autoregulation [17]. An internal deleted mutant of *c-myb* exon 9 gene has been found in an SBL tumor [12] with higher transcriptional activity than wild-type *c-myb*. To determine the type of *c-myb* mutant in SBL and EBL tumors, we used RT-PCR. However, the amplified *c-myb* specific DNA fragments were identical to the wild-type in size except for SBL No. 5, in which the DNA fragment was larger (400 bp) (Fig. 10). Molecular analysis of this fragment is now under way and the results will be published elsewhere. Further studies are needed to understand the tumorigenesis of SBL. These should include immunological and molecular investigations, including the expression of surface antigens, oncogenes and other activating genes.

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