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Differential Expression of Subspecies of Polyomavirus and Murine Leukemia Virus Enhancer Core Binding Protein, PEBP2, in Various Hematopoietic Cells

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The core sequence of the enhancer of murine leukemia virus (MuLV) long terminal repeat is highly conserved in a large number of MuLV strains and appears to play an essential role when SL3-3 or Moloney strains induce T cell lymphoma in mice. We found by using the electrophoretic mobility shift assay that a polyomavirus enhancer core-binding protein, PEBP2, bound to this core motif of MuLV. We also noted that PEBP2 in several hematopoietic cell lines derived from B lymphocyte, macrophage and myelocyte lineages migrated significantly faster than the authentic PEBP2 detected in NIH3T3 fibroblasts. Interestingly, PEBP2 detected in the cell lines of T lymphocyte lineage appeared to contain both types, which were indistinguishable in electrophoretic mobility from those of NIH3T3 and of B lymphocyte, macrophage and myelocyte lineages. The treatment of the nuclear extract containing PEBP2 with phosphatase generated PEBP3, which is a subcomponent of PEBP2 and retained the same DNA-binding specificity as PEBP2. The altered mobility of hematopoietic cell-derived or T lymphocyte-derived PEBP2 was found to be due to the alteration of the mobility of PEBP3. Based on the distinct mobility of PEBP2/3 of T lymphocytes from those of other hematopoietic cells, we discuss the implication of PEBP2 in MuLV-induced T cell leukemia and T cell-specific gene expression.

Key words: Polyomavirus — Murine leukemia virus — Enhancer core — T lymphocytes — PEBP2

A polyomavirus (Py) enhancer-binding protein 2 (PEBP2)[1,2] binds to both the A and B cores of the Py enhancer[3] (see Fig. 1). These two cores are essential elements of the Py enhancer in activating transcription as well as Py DNA replication.[4–8]

PEA2, equivalent to PEBP2, is undetectable in the murine embryonal carcinoma cell line, F9, but becomes detectable in differentiation-induced F9 cells and fibroblasts.[9,10] The PEA2/PEBP2-binding site in the A core is also included in the F9 cell-specific silencer (ΔF9-5000 element in Fig. 1), to which a ubiquitous repressor, PEBP4, binds.[9] The binding sites of these two factors overlap within the silencer. In undifferentiated F9 cells, PEBP4 binds to the ΔF9-5000 element and negatively regulates the Py early promoter function.[9] Upon differentiation, PEBP2 is induced, competes with PEBP4 for binding and converts the ΔF9-5000 element to a positively functioning element.[9] Therefore, PEBP2 is an activator protein. Unlike AP1 [PEA1[10,11] or PEBP1[1,2]] or Ets-related factor [PEA3[12] or PEBP5[13]],[14–19] PEBP2 responds poorly to 12-O-tetradecanoylphorbol-13-acetate and does not strongly stimulate transcription or Py DNA replication.[15,19]

NIH3T3 cells transformed by the activated c-Ha-*ras* oncogene do not contain PEBP2 but instead, contain a faster migrating factor PEBP3.[1] PEBP3 was subsequently found to bind to exactly the same sequence as PEBP2. PEBP2 can be converted to PEBP3 by treating the nuclear extract containing PEBP2 with phosphatase *in vitro* or by incubating cells expressing PEBP2 in the presence of protein kinase inhibitors.[2]

In the present study, we described another type of variability of PEBP2 in different hematopoietic cell types, particularly in T lymphocytes. We also demonstrated that PEBP2 bound to the enhancer core of murine leukemia virus (MuLV). The core of MuLV enhancer is one of the important sequence motifs regulating the tissue specificity of enhancer function.[20–26]

MATERIALS AND METHODS

Cells The mouse cell lines used were as follows: NIH3T3 fibroblasts, L1210 cells of B lymphocyte origin, S194 myeloma cells, EL4 and BW5147 cells of T lymphocyte origin, J774 and P388D1 cells of macrophage origin and the M1 myelocytic cell line. NIH3T3 and M1 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf or horse serum, respectively. EL4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The other cell lines were cultivated in DMEM supplemented with 10% fetal calf serum.

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Mobility shift assay The nuclear extract was prepared from tissue culture cells as described previously.[1] Complementary deoxyoligonucleotides representing the A core (nt 5107 through nt 5130) or the ΔF9-5000 element (nt 5119 through nt 5142) of the Py enhancer (Fig. 1) were synthesized with *Bam*HI and *Bgl*II cohesive ends. They were cloned into the *Bam*HI site of pUC13 vector so that the early or late gene side of the enhancer fragment was next to the *Eco*RI or *Hin*dIII site of the vector, respectively. The *Eco*RI-*Hin*dIII fragment harboring the insert was cleaved from the vector and its 3′ ends were labeled with ^{32}P. The binding reaction and the mobility shift assay were performed with ^{32}P-labeled probes (10 fmol, 20,000 cpm) using 10 μg protein of the nuclear extract as described previously.[1] Complementary deoxyoligonucleotides representing the enhancer core sequences of SL3-3 and Moloney MuLV strains (see Fig. 2A) were included as competitors in binding reactions. For phosphatase treatment, protein (10 μg) from the nuclear extract was incubated with 0.02 or 0.2 unit of potato acid phosphatase (Sigma Chemical Co., catalogue number, P-6760) for 30 min at room temperature, then the probe was added and the reaction mixture was processed for the mobility shift assay.

Methylation interference footprinting The 5′ ends of the *Eco*RI or *Hin*dIII sites of the fragment harboring the ΔF9-5000 element were asymmetrically labeled with ^{32}P. Purine residues in the fragment were methylated by dimethyl sulfate as described previously.[1] The methylated DNA probe was incubated with the nuclear extract of L1210 cells and processed for the mobility shift assay. The DNAs in the complexed and free forms were extracted from the gel and cleaved by reaction with piperidine as described previously.[9] DNA samples containing equal amounts of radioactivity per lane were loaded on an 8% polyacrylamide-urea sequencing gel.

RESULTS

Nomenclature of protein factors Since we will deal with several factors which have the same DNA-binding specificity but different mobility in gels, we will refer to the PEA2/PEBP2 site-binding factor detected in fibroblasts, hematopoietic cells except for T lymphocytes and

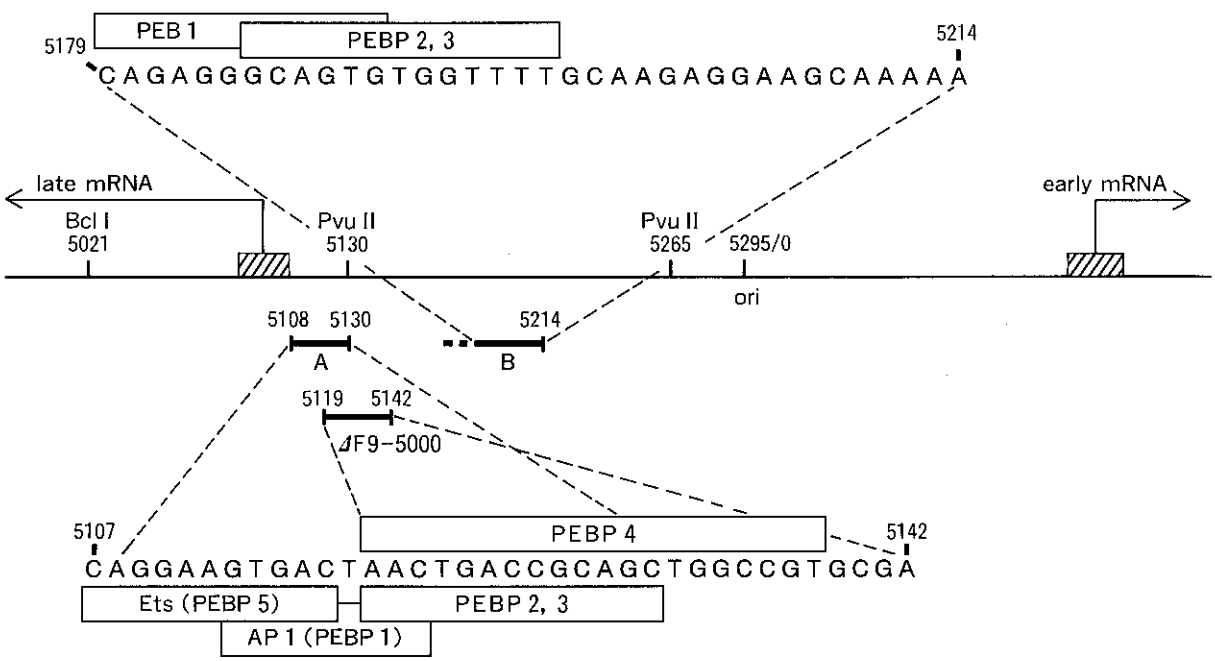

Fig. 1. Schematic illustration of the regulatory region of the Py genome. The hatched boxes represent the promoter region for transcription of the early and late genes. Ori means the origin sequence necessary for viral DNA replication. The enhancer is defined as the region spanning the *Bcl*I site (nt 5021) through the *Pvu*II site (nt 5265) and two elements, A and B, have been identified as the cores for enhancer function.[5,8] The A and B elements roughly correspond to the α and β cores reported by Muller *et al.*[7] The ΔF9-5000 element is the deleted region in the enhancer of a Py mutant, F9-5000.[49] The binding sites of each PEBP factor identified in our laboratory[1,2,9,13] are indicated. PEB1 is a factor reported by Piette and Yaniv.[50] The nucleotide sequence is shown in the early RNA strand sense.

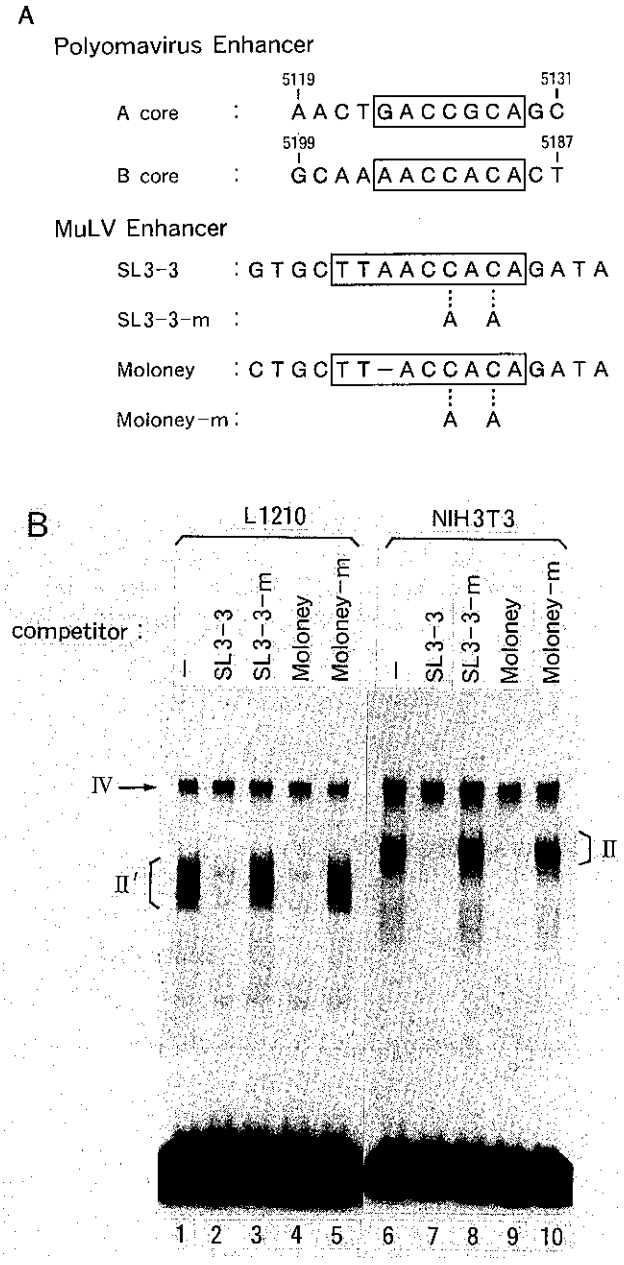

Fig. 2. Panel A. Nucleotide sequences showing the A and B cores of Py enhancer and the core motifs of MuLV enhancer. The boxed sequences in the A core (early RNA strand) and the B core (late RNA strand) represent the PEBP2/PEBP3-binding consensus. The oligonucleotides representing the core (boxed) and adjacent sequences of SL3-3 and Moloney strains and their mutated versions are shown. Panel B. Effect of the MuLV enhancer cores on the band shift of PEBP2. The nuclear extract of cells as indicated was incubated with the ^{32}P-labeled ΔF9-5000 element probe in the absence or presence of a 100-fold molar excess of unlabeled oligonucleotides shown on the top of the gels. The reaction mixture was processed for the mobility shift assay.

T lymphocytes as *f*PEBP2, *h*PEBP2, and *t*PEBP2, respectively. Likewise, PEBP3 from respective cell types will be called *f*PEBP3, *h*PEBP3 and *t*PEBP3.

Binding of PEBP2 to the MuLV enhancer cores The enhancer of MuLV is composed of several sequence motifs.[21] Some of these motifs including the core sequence are well conserved among many MuLV strains.[21] The core sequence can be classified into two groups (see Fig. 2A). The first is represented by the cores of SL3-3 strain (TTAACCACA), MCF247 strain (TCGACCACA), AKR strain (TTGACCACA) and Soule strain (TTGACCGCA) and the second by that of Moloney strain (TTACCACA). When the binding sites of PEBP2 in the Py enhancer A and B cores are compared with the core sequence of the MuLV enhancer, they are strikingly similar. The cores of the first group are perfectly compatible with the consensus binding sequence of PEBP2, PuACCPuCA,[3] whereas the Moloney type deviates slightly. We therefore examined whether PEBP2 would bind to the core motif of the MuLV enhancer.

In the mobility shift assay using the ^{32}P-labeled ΔF9-5000 element probe, the nuclear extract of NIH3T3 cells generated two complexes, IV and II (Fig. 2B, lane 6). Bands IV and II represent PEBP4 and PEBP2, respectively.[9] The extract of L1210 cells, of B lymphocyte origin, on the other hand, produced complex II′ that migrated significantly faster than complex II produced by the fibroblast nuclear factor (lane 1). Band II′ represents a PEBP2-like factor-DNA complex, as will be shown below, and it will be referred to as *h*PEBP2. PEBP2 from NIH3T3 fibroblasts will be termed *f*PEBP2. PEBP4 was detected in L1210 cells, and had the same mobility as that of fibroblasts.

The inclusion of 100-fold molar excess of unlabeled oligonucleotides harboring the core motif of SL3-3 or Moloney enhancer completely abolished the formation of bands II and II′ but not that of band IV (lanes 2, 4, 7 and 9). The use of the mutated MuLV core sequence as competitors (see Fig. 2A) did not affect the formation of complexes II and II′ (Fig. 2B, lanes 3, 5, 8 and 10). The results indicated that both *f*PEBP2 and *h*PEBP2 bound to the core sequences of not only the SL3-3 type but also the Moloney type. However, the binding affinity of both *f*PEBP2 and *h*PEBP2 to the core of Moloney enhancer was slightly lower than that to the SL3-3 enhancer core (data not shown). This must be due to the fact that the core sequence of the Moloney enhancer has a pyrimidine residue rather than a purine residue at the extreme 5′ position in the consensus sequence (see Fig. 2A). The biological significance of PEBP2 binding to the MuLV enhancer core will be discussed later.

The faster-migrating *h*PEBP2 recognizes the authentic PEBP2-binding sites in the Py enhancer Since PEA2/PEBP2 site-binding factor with different electrophoretic

mobility was detected in L1210 cells, this factor, *h*PEBP2, was characterized further. Also, various types of cells in the hematopoietic lineage including T lymphocytes were examined for the presence of novel PEA2/PEBP2 site-binding factor(s). First, to compare *f*PEBP2 and *h*PEBP2 more closely, the exact contact site of *h*PEBP2 on the ΔF9-5000 sequence of the Py enhancer was examined by the methylation interference footprinting method[9] (Fig. 3). The G residues at nt 5125, 5126 and 5128 on the late RNA strand interfered strongly with the binding of *h*PEBP2, whereas the G and A residues at nt 5121, 5122, 5131 and 5132 on the late RNA strand and those at nt 5123 and 5124 on the early RNA strand interfered weakly. We reported previously the footprint of *f*PEBP2 where only the G but not A residues were examined.[1] Comparing *h*PEBP2 and *f*PEBP2, their footprints were essentially the same. Namely, the methylated G residues at nt 5125, 5126 and 5128 on the late RNA strand and that at nt 5123 on the early RNA strand interfered with the binding of both *h*PEBP2 and *f*PEBP2.

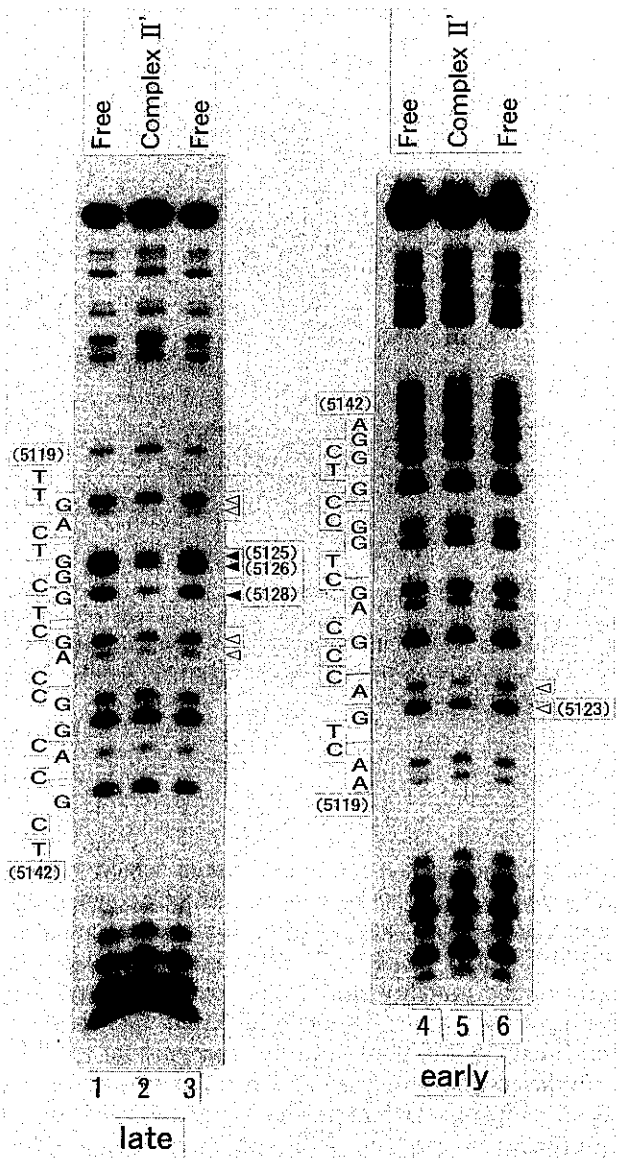

Fig. 3. Methylation interference footprinting of L1210 cell-derived *h*PEBP2 on the ΔF9-5000 element sequence. The complex II′ formed by incubating the nuclear extract of L1210 cells with the ΔF9-5000 element probe was footprinted on the late (lanes 1, 2 and 3) and early RNA strands (lanes 4, 5 and 6). DNA that formed the complex II′ (lanes 2 and 5) and DNA that migrated as free form (lanes 1, 3, 4 and 6) were run in parallel. The ΔF9-5000 element sequence is shown alongside the gel. The purine residues interfering strongly or weakly are indicated by closed or open triangles, respectively.

The nuclear factors which bind to the core sequences have been identified for strains of SL3-3,[25,26] Friend[27] and Moloney,[28] respectively. The reported methylation interference footprinting pattern of MuLV core binding factor on the MuLV enhancer is very similar to that of PEBP2 on the Py enhancer.

Conversion of PEBP2 to PEBP3 by phosphatase treatment When the nuclear extract of NIH3T3 cells was incubated with acid phosphatase and processed for the mobility shift assay using the A core probe, band II disappeared and band III appeared (Fig. 4, lanes 7, 8 and 9). As reported previously,[2] bands I, II and III represent

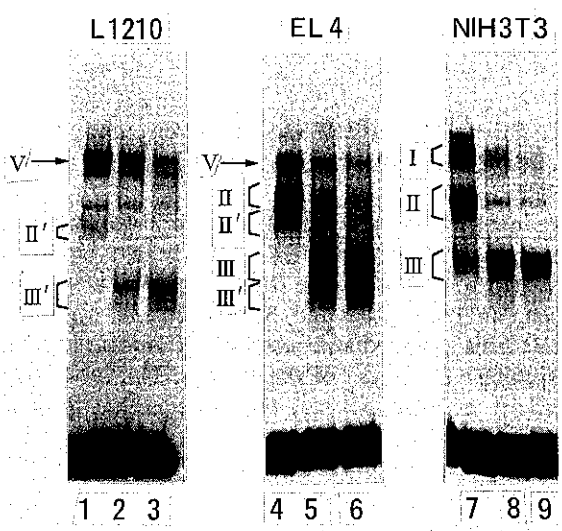

Fig. 4. Effect of phosphatase treatment on the band shift. The nuclear extract of L1210, EL4 or NIH3T3 cells was incubated without (lanes 1, 4 and 7) or with 0.02 unit (lanes 2, 5 and 8) or 0.2 unit (lanes 3, 6 and 9) of acid phosphatase and the reaction mixture was analyzed by mobility shift assay using the ^{32}P-labeled A core probe.

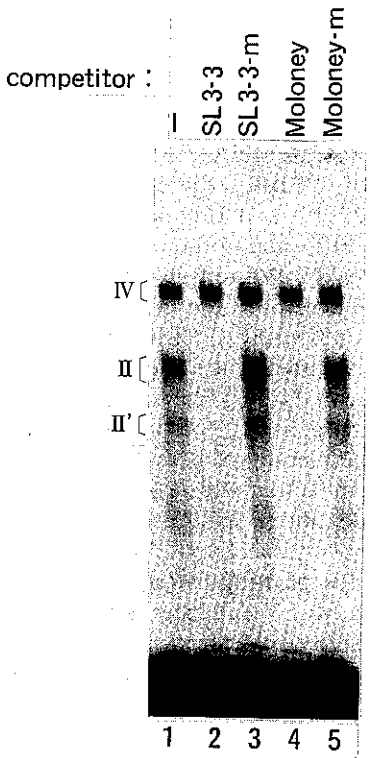

Fig. 5. Binding of *t*PEBP2 to the MuLV enhancer cores. The nuclear extract of EL4 cells was incubated with the ^{32}P-labeled ΔF9-5000 element probe in the absence or presence of a 100-fold molar excess of unlabeled oligonucleotides as shown on the top of the gel. The reaction mixture was analyzed by mobility shift assay.

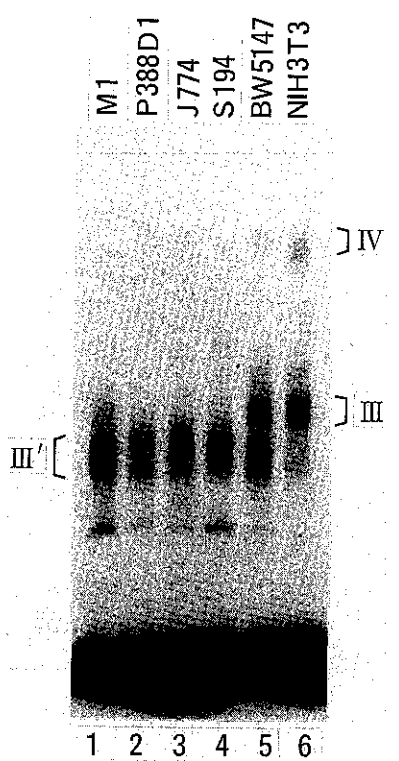

Fig. 6. PEBP3 activity expressed in several mouse cell lines of hematopoietic lineage. The nuclear extract of cells as indicated was incubated with acid phosphatase and the reaction mixture was analyzed by mobility shift assay using the ^{32}P-labeled ΔF9-5000 element probe.

AP1, PEBP2 and PEBP3, respectively, and PEBP3 in complex III retains the same DNA binding specificity as PEBP2.

We next examined whether *h*PEBP2 was molecularly related to authentic *f*PEBP2 and would similarly be converted to a PEBP3-like form by phosphatase. As can be seen in lanes 1, 2 and 3, complex II′ representing *h*PEBP2 disappeared and complex III′ appeared after the phosphatase treatment of the extract. The DNA-binding specificity of band III′ was the same as that of band III (data not shown). Therefore, the factor in L1210 cells responsible for band III′ was considered to be PEBP3-related and will be referred to as *h*PEBP3. We observed that complex II′ of L1210 cells migrated faster than complex II of NIH3T3 cells (compare lanes 1 and 7). A similar difference of mobility was noticed for bands III and III′ (compare lanes 3 and 9). The different mobility of *f*PEBP3 and *h*PEBP3 was also confirmed for the partially purified preparation on the heparin-Sepharose CL-6B (Pharmacia) column, through which both activities were eluted at the same 0.3 *M* KCl concentration (data not shown). The above observations suggested that *f*PEBP2/3 and *h*PEBP2/3, although migrating differently in the mobility shift assay, are related molecular entities rather than distinctly different factors having coincidentally the same sequence recognition.

In the case of the extract of T lymphocyte-derived EL4 cells, complexes II and II′ were both formed and the phosphatase treatment abolished both complexes and generated complexes III and III′ (Fig. 4, lanes 4, 5 and 6). The DNA-binding specificity of bands II, II′ and bands III, III′ of EL4 cells was the same as that of *f*PEBP2 and *f*PEBP3 (data not shown). We also confirmed that the formation of bands II and II′ of EL4 cells was abolished by including the unlabeled core sequence of SL3-3 or Moloney MuLV (lanes 2 and 4 in Fig. 5) but not by including the mutated core (lanes 3 and 5). PEBP2/3 in EL4 cells will be referred to as *t*PEBP2/3.

Table I. Possible PEBP2/3-binding Sites in the Enhancer of T Cell-specific Genes

Enhancer (binding element)		Sequence		Position
mouse TCR β	C A	A A C C A C T	A A	(201/191)
mouse TCR β	C A	A A C C A C A	T C	(635/625)
human TCR β (Tβ3)	C A	A A C C A C A	T C	(290/280)
mouse TCR β	G G	A A C C A C A	C T	(706/716)
human TCR β (Tβ4)	A G	A A C C A C A	C T	(365/375)
mouse TCR γ	T A	A A C C A C A	G C	(283/293)
mouse TCR γ	A A	G A C C A C A	G C	(314/324)
human TCR δ (δE3)	G A	A A C C A C A	T G	(1253/1243)
human TCR α (Tα4)	C C	A A C C G C A	G G	(191/201)
mouse CD3 δ	C T	T A C C A C A	G C	(15/25)
human CD3 ε (E2)	T T	A A C C A C A	G C	
human CD2 (E6)	C T	G A C C T C A	G G	(549/539)
PEBP2/3 consensus		PuACCPuCA		

The enhancer sequences of the genes expressed specifically in T lymphocytes were surveyed and those which are compatible with the PEBP2/3 binding consensus are listed. The references from which the enhancer sequences are quoted are as follows; the enhancer of mouse and human T cell receptor β chain gene from Fig. 4 of ref. 32 and from Fig. 3 of ref. 31, respectively, the enhancer of mouse T cell receptor γ chain gene from Fig. 4 of ref. 33, the enhancer of T cell receptor δ chain gene from Fig. 3 of ref. 34 and from Fig. 4 of ref. 35, the enhancer of T cell receptor α chain gene from Fig. 4 of ref. 36, the enhancer of CD3-δ gene from Fig. 4 of ref. 37, the enhancer of CD3-ε gene from Fig. 6 of ref. 38 and the enhancer of CD2 gene from Fig. 5 of ref. 39. The elements indicated by parenthesis represent the nuclear factor-binding sites reported in each reference. The location of the sequences are indicated by nucleotide numbers used in the respective figures in each reference. The nucleotide residues underlined deviate from the PEBP2/3-binding consensus.

In addition, the results in Fig. 4 indicated that AP1 in complex I and PEBP5 in complex V lost their DNA-binding activity after the phosphatase treatment, as observed before for AP1.[2] As for the consequence of dephosphorylation of AP1 on its DNA-binding activity, both positive and negative effects have been reported.[29, 30]

Expression of PEBP3 in other hematopoietic cell lines To survey whether hematopoietic cells generally express an anomalously migrating PEBP2-like factor, we examined several more cell lines for the expression of the PEBP3 activity (Fig. 6). The cell lines examined included Ml myelocytic cells, P388D1 and J774 macrophages and S194 myeloma cells. When the extract was incubated with phosphatase and processed for the mobility shift assay using the ΔF9-5000 element as a probe, all of the above cell lines generated the faster-migrating complex III′ (lanes 1 through 4). The exception was the extract of BW5147 cell line which is of T lymphocyte origin. This cell line produced both the slower-migrating complex III and the faster-migrating complex III′ (lane 5). The results showed that all five hematopoietic cell lines except for those of T cell origin expressed *h*PEBP3. The two T lymphocyte lines, EL4 and BW5147, expressed two factors indistinguishable in electrophoretic mobility from hematopoietic cell and fibroblast type PEBP3 (*t*PEBP3).

DISCUSSION

Wild-type Moloney or SL3-3 strains of MuLV cause T cell lymphoma when injected into newborn mice. The same viruses, but bearing a mutation solely in the enhancer core, have reduced T lymphomagenic activity and, in the case of Moloney strain, the mutant virus induces erythroleukemia after a longer latent period.[22, 23] The T lymphocyte-specific activity of the MuLV enhancer core has also been observed in transient expression assays.[20, 24–26] We have shown in this study that PEBP2 binds to the MuLV enhancer core and that T lymphocytes express *t*PEBP2 which is distinct in its electrophoretic mobility from *h*PEBP2 detected in other hematopoietic cells. We did not detect any factor in the T cell nuclear extract other than *t*PEBP2 which bound to the core of the MuLV enhancer. The results described in the present study, therefore, suggest that PEBP2, which is probably identical to the reported MuLV core-binding

factors,[25,26,28] is one of the major determinants of T cell tropism when the Moloney and SL3-3 strains induce leukemia.

A possible involvement of PEBP2 in T lymphocyte-specific gene expression was also noted when we surveyed the regulatory regions of cellular genes. Table I lists several sequence motifs which are compatible with the consensus PEBP2-binding site and which are found in the enhancer regions of the indicated genes. The genes regulated by these enhancers include T cell receptor β,[31,32] T cell receptor γ,[33] T cell receptor δ,[34,35] T cell receptor α,[36] CD3-δ gene,[37] CD3-ε gene[38] and CD2 gene,[39] all of which are expressed specifically in T lymphocytes. In the case of human T cell receptor β gene enhancer, base substitutions within the Tβ3 region drastically reduce T cell-specific function.[31] We confirmed that PEBP2 binds to the Tβ3 and Tβ4 regions (E. Ogawa, M. Satake and Y. Ito, unpublished observation).

We have shown previously that purified *f*PEBP3 is composed of α and β subunits, each of which further contains multiple species of polypeptides.[3] It is possible that the subunits (α or β) of PEBP2/3 or their subspecies are differentially expressed in various cell lines, giving rise to multiple forms of PEBP2/3 observed in this study. Therefore, the subunits or subspecies of *t*PEBP2/3 which are expressed specifically in T lymphocytes probably contribute to the T cell-specific gene expression.

As for the exact molecular nature of *f*-, *h*- and *t*-PEBP2, one can imagine several possibilities. For example, each of them may be encoded by different but related genes which are expressed differentially in various cell types, as in the case of the family of octamer-binding factors, Oct 1, Oct 2 and Oct 3.[40-47] Another possibility would be that a transcript from one gene is alternatively spliced in a tissue-specific manner. Multiple species of CCAAT-box-binding proteins, CTF, are encoded in the same gene but are generated by differential splicing and are all capable of binding to the same recognition sequence.[48] A differential posttranslational modification of the factor, such as phosphorylation or proteolytic cleavage, may also explain the different mobilities of various forms of PEBP2. cDNA cloning of the PEBP2 gene(s) is under way and structural analysis will eventually elucidate the molecular basis of the various forms of PEBP2 in different cell types.

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