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Expression of the Runt Domain-Encoding *PEBP2α* Genes in T Cells during Thymic Development

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The *PEBP2αA* and *PEBP2αB* genes encode the DNA-binding subunit of a murine transcription factor, PEBP2, which is implicated as a T-cell-specific transcriptional regulator. These two related genes share the evolutionarily conserved region encoding the Runt domain. *PEBP2αB* is the murine counterpart of human *AML1*, which is located at the breakpoints of the 8;21 and 3;21 chromosome translocations associated with acute myeloid leukemia. Northern (RNA) blots of various adult mouse tissues revealed that the levels of expression of both genes were most prominent in the thymus. Furthermore, transcripts of *PEBP2αA* and mouse *AML1*/*PEBP2αB* were detected in T lymphocytes in the thymuses from day 16 embryos and newborns, as well as 4-week-old adult mice, by in situ hybridization. The expression of the genes persisted in peripheral lymph nodes of adult mice. The transcripts were detected in all the $CD4^-$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^+$ $CD8^-$, and $CD4^-$ $CD8^+$ cell populations. The results indicated that both genes are expressed in T cells throughout their development, supporting the notion that PEBP2 is a T-cell-specific transcription factor. Transcripts of mouse *AML1*/*PEBP2αB* were also detected in day 12 fetal hematopoietic liver and in the bone marrow cells of newborn mice. The implication of mouse *AML1*/*PEBP2αB* expression in hematopoietic cells other than those of T-cell lineage is discussed in relation to myeloid leukemogenesis.

It is believed that T-lymphocyte-specific gene expression is achieved by the cooperation of a set of several transcription factors (see reference 22 for a review). Some of these are expressed specifically or mainly in T cells, whereas others are ubiquitously expressed in various tissues. GATA3 (12, 16, 20, 24, 47), TCF1/LEF-1 (33, 40, 41, 44, 45), Ikaros (6), and Ets-1 (11) are among those belonging to the former category, and the CREB/ATF family is of the latter (8). Their binding sequences have been identified in the enhancer elements of T-cell-specific genes such as the CD3δ (6) and T-cell receptor (TCR) genes (22).

We recently reported that the murine transcription factor PEBP2 is likely to be an important member of such transcription factors involved in T-cell-specific gene expression (30, 32). PEBP2 was originally identified as a polyomavirus enhancer-binding protein which interacts with both the A and B cores (18, 35). It is undetectable in F9 embryonal carcinoma cells and becomes detectable as the cells are induced to differentiate, suggesting that it is likely to be involved in the regulation of early mouse embryo development (5). Structurally, it is a heterodimer composed of α and β subunits (18). The α subunit bears a sequence-specific DNA-binding activity and recognizes the consensus sequence, Pu/TACCPuCA (32). The β subunit enhances the DNA-binding affinity of the α subunit through heterodimer formation (30). A 128-amino-acid region of the α subunit is highly homologous to the corresponding region of the *Drosophila* segmentation gene *runt* (19). The homologous region is termed the Runt domain, and it harbors the DNA-binding and heterodimerizing activities (17, 32). There are two related but independent genes, *PEBP2αA* and *PEBP2αB*, which encode the Runt domain (2, 32).

The notion that PEBP2 is likely to be involved in tissue-specific transcriptional regulation in the T-lymphocyte lineage is based on the following observations. First, Northern (RNA) blots of the RNA revealed that *PEBP2αA* was expressed at high levels in T-cell lines, whereas it was undetectable in the B-cell lines tested (32). Transcripts of the related gene, *PEBP2αB*, were also detected in the T-lymphocyte line as well as in some B-cell lines (2). Although the expression of these genes along with stages of differentiation of T and B cells has not been studied, it is noteworthy that they are expressed in all the T-cell lines tested so far. Consistent with this observation, there is a PEBP2 site in the regulatory regions of many T-cell-specific genes, including the TCR genes α, β, γ, and δ (36). The αA and αB proteins, when exogenously expressed, indeed bind to the PEBP2 site of the Tβ3 and Tβ4 elements in the TCRβ enhancer and stimulate the enhancer activity through the PEBP2 site (1, 32).

PEBP2 binds to the enhancer core sequence of murine leukemia virus (36). The wild-type Moloney or SL3-3 strain induces T-cell lymphoma when injected into newborn mice, whereas viruses carrying mutations in the enhancer core cause erythroleukemia after a prolonged latent period (9, 37). Nuclear factors called core-binding factor (CBF) or SL3-3 enhancer factor 1 are equivalent to PEBP2 in their core-binding activity (39, 42). Indeed the cDNA sequence of the CBF β-subunit gene (43) is identical to that of the *PEBP2β* gene (30).

Recent studies have revealed a notable aspect of the T-cell-specific function of PEBP2/CBF. In the TCRβ enhancer, PEBP2/CBF-binding sites in the Tβ3 and Tβ4 (or βE2 and

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βE3) elements are present near the regions where Ets-1 binds (22). The binding of these two factors is cooperative (46). Similarly, PEBP2/CBF and c-Myb cooperate for the T-cell-specific expression of TCRδ (10). While relative spacing and orientation of the PEBP2/CBF and Ets-1 sites are not important in the TCRβ enhancer, the restriction on the spacing has to be much tighter between the PEBP2/CBF and c-Myb sites in the TCRδ enhancer for cooperation (10, 46). These results suggest that PEBP2/CBF may indeed be involved in a combinatorial effect of several transcription factors to exert T-cell-specific transcription activation.

The possibility that PEBP2 also plays an important role in myeloid cells has been suggested in a remarkable way. The human counterparts of both *PEBP2αB* and *PEBP2*β, which encode entirely different polypeptides, are located at the breakpoints of chromosome translocations characteristic of acute myeloid leukemia (AML). The human gene *AML1*, located on chromosome 21q22, is identified at the breakpoints of t(8;21) AML (4, 26, 27) and t(3;21) therapy-related leukemia (25, 29). *PEBP2αB* is the mouse homolog of *AML1* (1, 2). The human gene encoding PEBP2/CBFβ is located at the breakpoints of the inversion of chromosome 16 (23), and *inv* (16) is a karyotypic abnormality of another subtype of AML (21). *AML1* has been shown by Northern blotting to be expressed in human bone marrow cells (26).

In this study, we examined first whether the in vivo expression of *PEBP2αA* and mouse *AML1*/α*B* (m*AML1*/α*B*) supports the notion that PEBP2/CBF is a T-cell-specific transcription factor. The results indicated that both genes are expressed at the highest levels in thymuses of adult mice and that their transcripts are present in thymocytes throughout thymic development. The expression of m*AML1*/α*B* in hematopoietic cells other than T cells was also examined in relation to myeloid leukemogenesis.

MATERIALS AND METHODS

RNA extraction and Northern blotting. Four-week-old male ICR and nude mice were purchased from Shimizu Experimental Materials (Kyoto, Japan). The animals were sacrificed under ether anesthesia, and then the fresh organs were quickly removed and frozen in liquid nitrogen. The organs were transferred into denaturing solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), homogenized in a Polytron, and then sonicated on ice. RNAs were extracted with acid-guanidinium thiocyanate-phenol-chloroform (3). Poly(A)$^+$ RNAs were selected by passage through an oligo(dT)-cellulose column once. A 2.5-μg aliquot of poly(A)$^+$ RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N membrane (Amersham) in 20× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate). The probes used were as follows: the *Nco*I (nucleotide [nt] 1293)-to-*Hin*dIII (nt 1688) fragment of the *PEBP2αA* cDNA (32), nt 1 to 1778 of the m*AML1*/*PEBP2αB* cDNA sequence (2), and the *Eco*RI (nt 1)-to-*Hin*dIII (nt 1050) fragment of the *PEBP2*β cDNA (30). The blot was hybridized with ^{32}P-labeled probes (10^6 cpm/ml) in a mixture containing 50% formamide, 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 5× SSC, 50 mM sodium phosphate (pH 7.0), and 100 μg of denatured salmon sperm DNA per ml at 42°C for 16 h. The blot was washed once in 2× SSC–0.1% SDS for 30 min at room temperature and twice in 0.1× SSC–0.1% SDS for 30 min each time at 60°C and exposed to X-ray films.

Probes used for in situ hybridization. Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared by using the DIG RNA labeling kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions. To generate the *PEBP2αA* probe, for example, the *Mlu*I (nt 3991)-to-*Eco*RI (nt 4662) fragment of the *PEBP2αA* cDNA (32) was subcloned into the *Sma*I and *Eco*RI sites of the Bluescript I pSK(−) plasmid. This plasmid (1 μg) was either linearized with *Bam*HI and transcribed with T7 RNA polymerase to generate an antisense probe or linearized with *Eco*RI and transcribed with T3 RNA polymerase to generate a sense probe. The other cDNA sequences used for generating RNA probes were as follows: the *Not*I (nt 1250)-to-*Hin*dIII (nt 1688) fragment of *PEBP2αA* cDNA (32), the *Dra*II (nt 228)-to-*Bst*EII (nt 560) fragment of m*AML1*/*PEBP2αB* cDNA (2), the *Bsi*EI (nt 395)-to-*Sau*3A (nt 807) fragment of m*AML1*/*PEBP2αB*, the *Bam*HI (nt 1038)-to-*Dra*II (nt 1350) fragment of m*AML1*/*PEBP2αB*, the *Dra*II (nt 1350 to 1670) fragment of m*AML1*/*PEBP2αB*, and the *Bam*HI (nt 928 to 1744) fragment of murine *GATA3* cDNA (20). All these cDNA sequences were subcloned in pSK(−), linearized with appropriate enzymes, and transcribed by either T7 or T3 RNA polymerase. After transcription, 40 U of RNase-free DNase was added to the reaction mixture, and the mixture was incubated for a further 10 min at 37°C. Transcription products were ethanol precipitated and dissolved in distilled water.

In situ hybridization histochemistry. Hybridization proceeded as described (28).

(i) Tissue preparation. The mouse organs were fixed with fresh 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0 (PB), at 4°C for 16 h. They were successively dehydrated with 70, 80, 90, and 99.5% ethanol and then embedded in paraffin. Sections were cut on a microtome and mounted on 3-(triethoxylosilyl)-propylamine (Merck, Schuchardt, Munich, Germany)-coated glass slides. They were stored at 4°C until use.

(ii) Preparation of the sections. After the sections were dried, the paraffin was removed by successively immersing the sections in xylene and 99.5, 90, 80, and 70% ethanol. They were rinsed once in PB and postfixed with 4% paraformaldehyde in PB for 15 min. After being rinsed in PB, the sections were incubated with 1 μg of proteinase K (Boehringer Mannheim) per ml in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA for 30 min at 37°C. They were fixed again in 4% paraformaldehyde in PB for 10 min. After a rinse with PB and equilibration with 0.1 M triethanolamine-HCl buffer (pH 8.0), the sections were acetylated with fresh 0.25% acetic anhydride in 0.1 M triethanolamine-HCl buffer (pH 8.0) for 10 min. They were dehydrated by passage through PB and 70, 80, 90, and 99.5% ethanol and then air dried.

(iii) Hybridization and washing. The hybridization solution contained 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS, 10 mM Tris-HCl (pH 7.6), 250 μg of *Escherichia coli* tRNA per ml, 10 mM dithiothreitol and an appropriately diluted digoxigenin-UTP-labeled RNA probe. Hybridization was carried out at 50°C for 16 h in a moisture chamber. After hybridization, the sections were preincubated in 5× SSC at 50°C and then in 50% formamide–2× SSC at 50°C for 30 min to remove excess probe. They were then incubated in TNE (10 mM Tris-HCl [pH 7.6], 500 mM NaCl, 1 mM EDTA) buffer for 10 min and with 10 μg of RNase A per ml in TNE buffer for 30 min at 37°C. To remove digested RNA, the slides were incubated in 2× SSC for 20 min once and in 0.2× SSC for 20 min twice at 50°C.

(iv) Immunodetection of hybridized probe. The sections were incubated in DIG buffer 1 (100 mM Tris-HCl [pH 7.5], 150 mM NaCl) for 2 min and then with 1.5% blocking reagent in DIG buffer 1 for 60 min. They were next incubated in polyclonal sheep antidigoxigenin Fab fragment diluted 1:500 in DIG buffer 1 for 60 min. The sections were washed with DIG buffer 1 for 15 min twice and equilibrated with DIG buffer 3 (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2$). The color was developed by incubating the sections with 337.5 μg of nitroblue tetrazolium and 165 μg of 5-bromo-4-chloro-3-indolyl phosphate toluidinium (BCIP) in DIG buffer 3 at 37°C until the signal/noise ratio reached a maximum. The reaction was stopped by rinsing the sections with a 10 mM Tris-HCl (pH 8.0)–2 mM EDTA solution. The slides were mounted with Crystal Mount (Biomeda Corp., Foster City, Calif.). If necessary, the coverslips were dislodged by immersing the slides in water for several days and the sections were stained with hematoxylin.

Cell sorting. Thymocytes obtained from 4-week-old C57BL/6 mice were stained with fluorescein isothiocyanate-conjugated monoclonal antibody to CD4 (Rm4-5; Pharmingen, San Diego, Calif.) and biotinylated monoclonal antibody to CD8 (53-6-72; Becton Dickinson Immunocytometry Systems, San Jose, Calif.) followed by phycoerythrin-labeled streptavidin (Bethesda Research Laboratories, Gaithersburg, Md.). The cells were stained in the presence of a monoclonal antibody to the Fcγ receptor (2.4G2) to block nonspecific binding via the Fcγ receptor. CD4$^-$ CD8$^-$, CD4$^+$ CD8$^+$, CD4$^-$ CD8$^+$, and CD4$^+$ CD8$^-$ populations of thymocytes were electronically sorted by using a FACS Vantage cell sorter (Becton Dickinson). Cells were sorted in the presence of propidium iodide to exclude dead cells. The purity of each population was 98% in this study.

RESULTS

Northern blot analysis of the transcripts of the *PEBP2*α and -β genes. Poly(A)$^+$ RNAs prepared from various organs of 4-week-old adult mice were analyzed by Northern blotting (Fig. 1). As an internal control, the β-actin gene was expressed (Fig. 1D), which indicated that relatively uniform amounts of RNAs were loaded in the lanes in Fig. 1.

PEBP2αA transcripts of 4.7 and 2.1 kb were detected in the thymus, whereas only a 2.4-kb transcript was observed in the testis. No other tissues tested expressed α*A* at a detectable level. As reported previously (32), much larger transcripts (7.4- and 6.3-kb RNAs) were detected in mouse T-cell lines EL4 and BW5147 as well as in NIH 3T3 fibroblasts. The significance of the difference in the size of the α*A* transcripts is not clear at

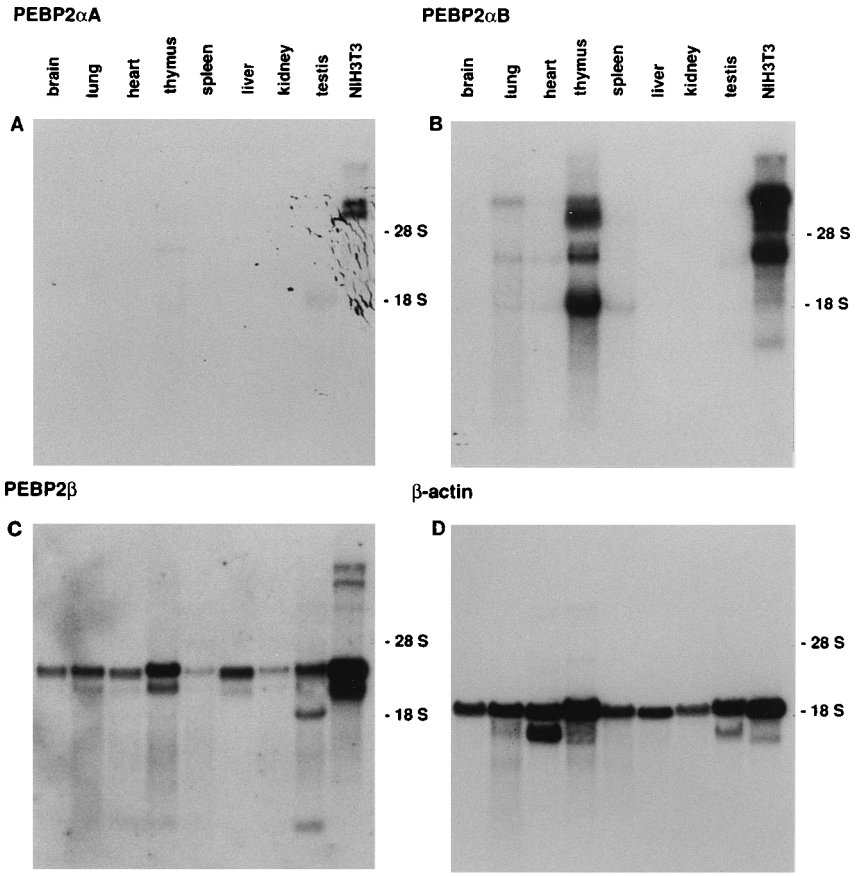

FIG. 1. Northern blot analysis of the transcripts of the *PEBP2* genes in various tissues. Poly(A)$^+$ RNA was extracted from various tissues of 4-week-old male mice and separated on an agarose gel containing formaldehyde. The blots were hybridized with the indicated probes. Locations of the 28S and 18S rRNAs are indicated.

present. The levels of expression of α*A* in thymus and testis were lower than those in NIH 3T3 cells.

The four major sizes of m*AML1*/*PEBP2*α*B* transcripts, 7.9, 6.2, 3.8, and 2.1 kb, were detected in the thymus (Fig. 1B). RNAs of similar sizes have been detected in T-cell lines (2). Relatively small amounts of the 7.9-, 3.8-, and 2.1-kb transcripts were detected in the lung, heart, and testis. In the spleen, only the 2.1-kb transcript was evident, and its significance will be described later. A subset of the transcripts, 7.9- and 3.8-kb RNAs, were detected in NIH 3T3 cells as reported previously (2). With respect to the relative amounts of RNAs, comparable amounts of the m*AML1*/α*B* transcripts were detected in the thymus and NIH 3T3 cells.

These results indicated that the expression of α*A* and m*AML1*/α*B* is tissue type dependent and that the thymus is one of the main tissues expressing both α*A* and m*AML1*/α*B* in abundance. In contrast, the 3.0- and 2.5-kb transcripts of *PEBP2*/*CBF*β were detected in all the tissues examined at relatively uniform levels (Fig. 1C). This finding is analogous to that showing the ubiquitous expression of *PEBP2*/*CBF*β in various types of cultured cells (30). The two sizes of RNAs are likely to be due to alternative polyadenylations as shown in the RNAs obtained from NIH 3T3 cells (30). The nature of the two additional smaller transcripts in the testis is unclear at present. The expression profile of *PEBP2*/*CBF*β shown in Fig. 1 is in general agreement with that reported by others (43).

***PEBP2*α expression in the thymus and lymph nodes as detected by in situ hybridization.** The thymus, along with neighboring tissues, was dissected from day 16 mouse embryos, and the expression of α*A* and m*AML1*/α*B* was examined by means of in situ hybridization (Fig. 2). Expression of the gene for the known T-cell-specific transcription factor GATA3 was examined in parallel. Weak but positive signals were detected in the thymus by the α*A* probe (Fig. 2A). Much stronger signals were detected by the m*AML1*/α*B* and *GATA3* probes in the thymus (Fig. 2B and C, respectively). On fetal day 12, T-cell precursors are homing to the thymus, and surface expression of the αβ TCR proteins starts to be detected on day 16. If the positive signals in the thymus (Fig. 2A and B) are indeed due to reactions in the thymocytes themselves, the result suggests that α*A* and m*AML1*/α*B* are expressed during a relatively early period of T-cell development.

Results similar to those described above were obtained for the thymuses taken from newborn mice: a weak but positive signal for α*A* and a strong signal for m*AML1*/α*B* and *GATA3*. The staining profile of m*AML1*/α*B* is shown in Fig. 3A as an example. When viewed at a higher magnification (Fig. 3B), most of the cells are positive for the signal. By microscopic observation of the identical section after hematoxylyn staining, the cells positive for the signal were found to be small, containing little cytoplasm with densely stained nuclei, all of which are morphological characteristics of lymphocytes (data not shown). It is likely, therefore, that the cells positive for the m*AML1*/α*B* expression represent T lymphocytes rather than stromal cells.

In the thymuses of 4-week-old adult mice (Fig. 4), the α*A*

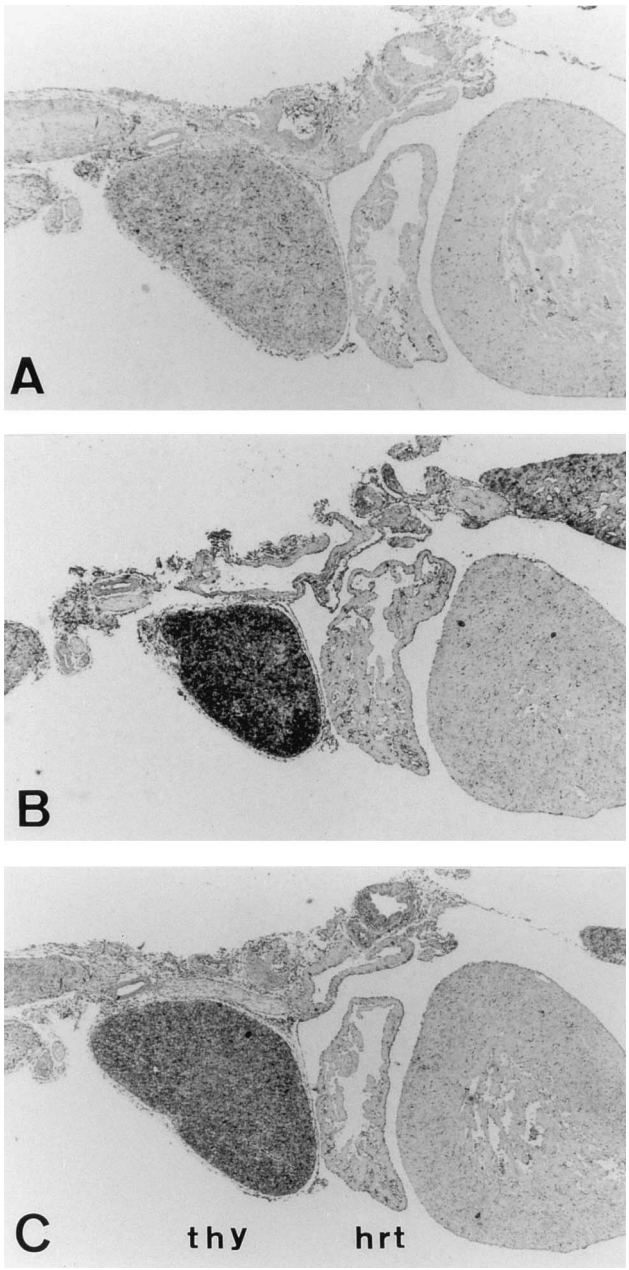

FIG. 2. Detection of the transcripts of *PEBP2αA* (A), m*AML1*/*PEBP2αB* (B), and *GATA3* (C) in day 16 fetal thymus by means of in situ hybridization. thy, thymus; hrt, heart.

signal became prominent compared with that from fetal thymus (Fig. 4A). The signals of m*AML1*/*αB* (Fig. 4B) and *GATA3* (Fig. 4C) remained intensely positive. The signals from the m*AML1*/*αB* transcripts appeared to be stronger in the cortex than in the medulla in the adult thymus (Fig. 4B), although the data were of a qualitative, not a quantitative, nature. Since the cortex is the site where T cells rapidly proliferate and undergo positive selection, the expression of *αA* and m*AML1*/*αB* may be correlated with cell proliferation. After maturation, T cells enter the medulla and are then distributed to peripheral organs such as the lymph nodes. In those from the inguinal region of adult mice, a strong signal was detected for m*AML1*/*αB* (Fig. 5B), whereas the *αA* (Fig. 5A) and *GATA3* (Fig. 5C) probes gave moderate signals.

In all of the studies shown in Fig. 2 through 5, the specificity of the positive signals was confirmed by the following criteria. (i) The antisense probes gave the signals, but the corresponding sense probes did not. For example, the sense probe for m*AML1*/*αB* did not generate signals in the thymus (Fig. 4D) or lymph nodes (Fig. 5D). (ii) The antisense probes that represented different portions of the respective cDNA sequences gave the same staining profile. For the *αA* or m*AML1*/*αB* probe, two or four different parts of the cDNA sequences, respectively, were selected to synthesize antisense probes. Among those, the sequences spanning nt 1250 through 1688 of *αA* cDNA and nt 228 through 807 of m*AML1*/*αB* cDNA contained the Runt domain-encoding regions (see Materials and Methods for the nucleotide numbers). On the other hand, the sequences spanning nt 3991 through 4662 of *αA* and nt 1038 through 1670 of m*AML1*/*αB* did not contain the Runt domain-encoding regions. They had no homology. Therefore, the signals detected by the unique region probes must have represented the specific transcripts of *αA* and m*AML1*/*αB*. The low level of *αA* signals, therefore, was not due to the cross-hybridization of the probe with the transcripts of m*AML1*/*αB*. Ribonucleotide probes representing the unique regions of *αA* and m*AML1*/*αB* (nt 3991 through 4662 of *αA* and nt 1038 through 1670 of m*AML1*/*αB*) were used in all the in situ hybridization experiments described herein. (iii) Cultured COS7 cells were transfected with the plasmid expressing *αA* or m*AML1*/*αB* and processed for in situ hybridization. Only the antisense probe generated signals in transfected cells but not in untransfected cells (data not shown).

***PEBP2α* expression in sorted thymocytes.** Thymocytes were prepared from the thymuses of adult mice and sorted into four subpopulations by using the surface markers CD4 and CD8. The cell pellets from each subpopulation were processed for in situ hybridization. As shown in Fig. 6, the *αA* transcripts were detected in $CD4^-$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^+$, and $CD4^+$ $CD8^-$ subpopulations by the antisense probe, whereas the sense probe did not produce positive signals. The antisense but not the sense probe produced positive signals for m*AML1*/*αB* in all subpopulations as well (data not shown). These results established that the cells expressing *αA* and m*AML1*/*αB* in the thymus represent T lymphocytes and that both *αA* and m*AML1*/*αB* are expressed in T cells regardless of the expression of CD4 and CD8 molecules.

Expression of m*AML1*/*PEBP2αB* in fetal liver and in bone marrow cells. The result of the Northern blot analysis of various tissues shown in Fig. 1 revealed that m*AML1*/*αB* is also expressed in the spleen, although more weakly than in the thymus. This raised the question as to whether the cells in the spleen which express m*AML1*/*αB* are T cells or of other lineages. As shown in Fig. 7, the 2.1-kb transcript of m*AML1*/*αB* was detected by Northern blotting in samples of the spleens taken from nude mice. In this case, the level of expression of m*AML1*/*αB* was comparable to that in the thymuses from normal mice. At the moment, the significance of the apparent difference in the level of the m*AML1*/*αB* expression in the spleen between normal and athymic mice is unclear. The significance of the predominant expression of the 2.1-kb transcript of m*AML1*/*αB* in spleen cells or the structure of mAML1/αB protein encoded by the 2.1-kb transcript is also unclear at present. T lymphocytes are excluded from the hematopoietic cells in nude mice, while erythroblasts and B lymphocytes constitute a major population of hematopoietic spleen cells. Clearly, these or some other spleen cells different from the T-cell population must also express m*AML1*/*αB*.

FIG. 3. Detection of the transcripts of m*AML1/PEBP2αB* in the thymus from a newborn mouse by means of in situ hybridization. Lower (A) and higher (B) magnifications are shown.

We further investigated the expression of m*AML1*/α*B* in fetal liver and bone marrow. Poly(A)$^+$ RNA was prepared from the livers of day 12 embryos and hybridized with the m*AML1*/α*B* probe. The sizes of the m*AML1*/α*B* transcripts were similar to those in the adult thymus (Fig. 7). This is in contrast to the lack of expression of m*AML1*/α*B* in the liver of adult mice (Fig. 1). We determined by means of in situ hybridization whether hepatocytes or hematopoietic cells in the liver of the day 12 embryo express m*AML1*/α*B*. There were positive signals detected for m*AML1*/α*B* (Fig. 8A). Some examples of positive cells are indicated by arrows. After hybridization, the same section was stained with hematoxylin (Fig. 8B). We compared the corresponding cells in a set of stained sections and found that large cells with lightly stained nuclei that represent primitive hepatocytes were negative for the signal. This is consistent with the result shown in Fig. 1, indicating that liver cells do not express m*AML1*/α*B*. The cells possessing densely stained nuclei are of hematopoietic lineage. A significant proportion of these cells were positive for the signal. A large population of cells of hematopoietic lineage in the fetal liver are erythroid (14). The results shown above indicated that other hematopoietic cells in addition to T lymphocytes express m*AML1*/α*B*.

Bone marrow cells were prepared from the ribs of newborn mice, and the expression of m*AML1*/α*B* was examined by means of in situ hybridization (Fig. 9A). The same section was stained with hematoxylin after hybridization (Fig. 9B). The positive cells (indicated by arrows) were large and contained large and lightly stained nuclei. They were considered to represent either erythroblasts or myeloblasts. Typical mature granulocytes that possessed doughnut-shaped nuclei were negative for the signal. These results indicated that m*AML1*/α*B* is expressed in either erythroblasts or myeloblasts or both.

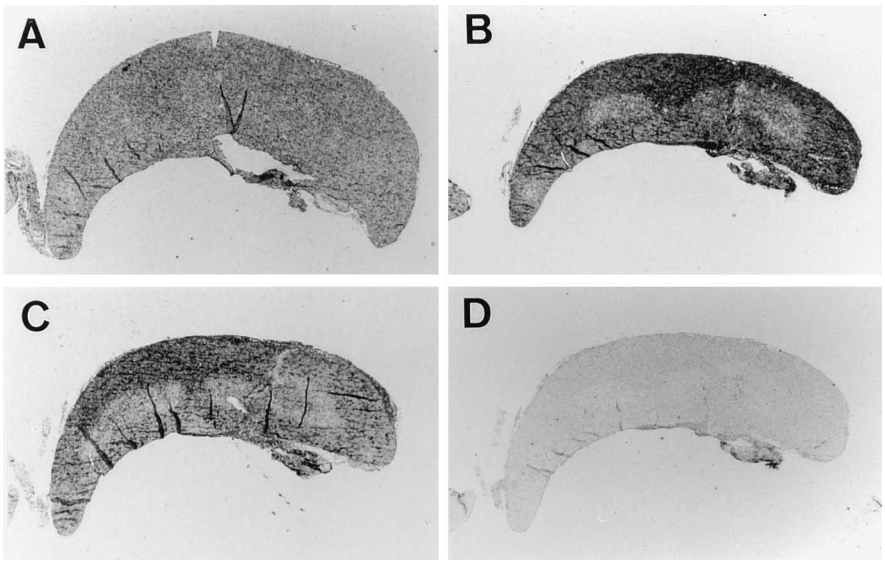

FIG. 4. Detection of the transcripts of *PEBP2αA* (A), m*AML1/PEBP2αB* (B), and *GATA3* (C) in the thymus from an adult mouse by in situ hybridization. In panel D, a sense probe for m*AML1/PEBP2αB* was used.

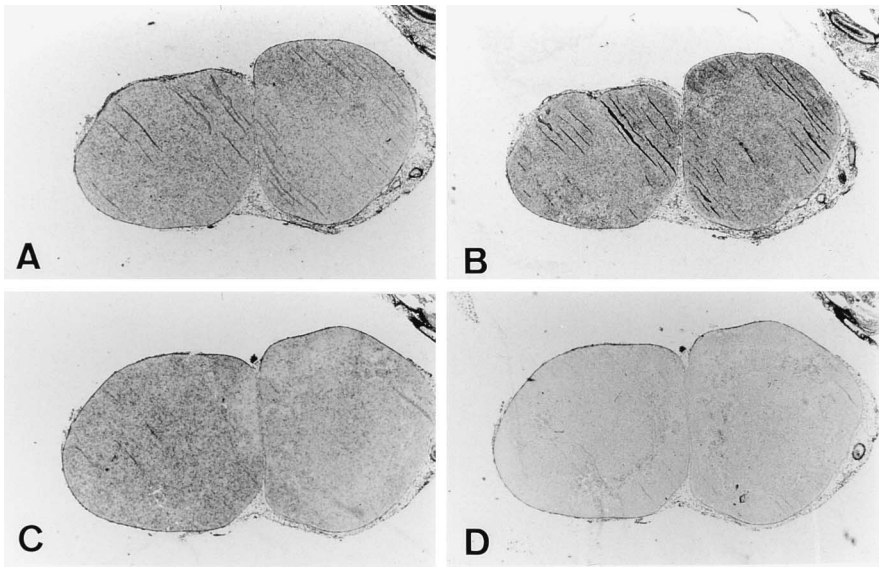

FIG. 5. Detection of the transcripts of *PEBP2αA* (A), m*AML1*/*PEBP2αB* (B), and *GATA3* (C) in an inguinal lymph node from an adult mouse by means of in situ hybridization. (D) A sense probe for m*AML1*/*PEBP2αB* was used.

DISCUSSION

This study showed that both the *PEBP2αA* and m*AML1*/*αB* genes are expressed throughout thymic T-cell development and in all of the T-cell subpopulations tested. These results are consistent with our conclusion that PEBP2 is involved in the regulation of T-cell-specific gene expression (32).

It is generally believed that the T-cell-specific transcriptional regulation is achieved by means of the combinatorial effect of a set of T-cell-specific and more general transcription factors (22). From this premise, it is important to examine whether PEBP2/CBF cooperates with other transcription factors, especially with those that are known to be T-cell specific. PEBP2/CBF stabilizes the binding of Ets-1 to DNA by binding to the PEBP2/CBF site located near that of Ets-1 site in the TCRβ enhancer (46). This notion of cooperativity of the in vitro binding is further strengthened by the results showing that the

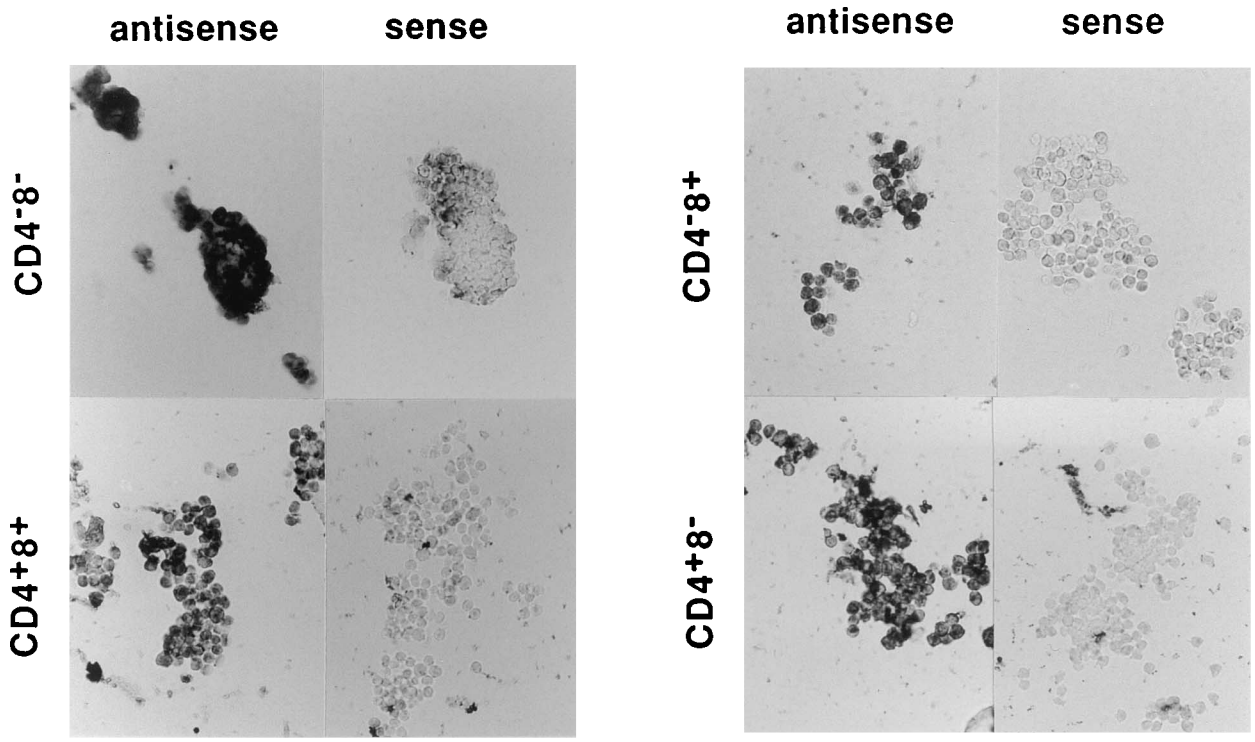

FIG. 6. Detection of the transcripts of *PEBP2αA* in sorted thymocytes. $CD4^-$ $CD8^-$, $CD4^+$ $CD8^+$, $CD4^-$ $CD8^+$, and $CD4^+$ $CD8^-$ thymocytes were processed for in situ hybridization, using antisense and sense probes.

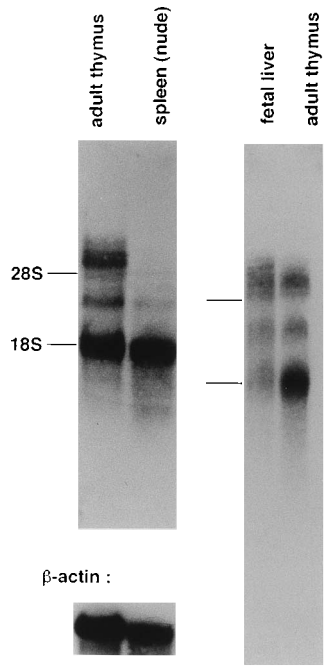

FIG. 7. Northern blots of the m*AML1*/*PEBP2*α*B* expression in spleen from nude mice and fetal liver of ICR mice. Poly(A)$^+$ RNA was prepared from spleens of adult nude mice and from day 12 fetal livers of ICR mice and separated on an agarose gel containing formaldehyde. Poly(A)$^+$ RNA from the thymuses of adult ICR mice was run in parallel. The blot was hybridized with the m*AML1*/*PEBP2*α*B* probe. Locations of the 28S and 18S rRNAs are indicated.

two factors cooperatively stimulated transcription through the Tβ3 and Tβ4 elements of the TCRβ enhancer in vivo (31). Ets-1 functionally cooperates with another lymphoid-specific factor, LEF-1, in the TCRα enhancer in a context-dependent manner (7). It will be of interest to examine the relationship among PEBP2/CBF, Ets-1, and LEF-1 that results in cooperativity in the T-cell-specific transcriptional regulation.

The expression profile of α*A* and m*AML1*/α*B* in the thymus was quite similar to that of the *GATA3* gene. Transcripts of all of them were detected throughout the T-cell ontogeny examined. GATA3 binds to the Tα3 element of the TCRα enhancer (12), to the Tβ2 element of the TCRβ enhancer (24), and to the δE1 and δE4 sites of the TCRδ enhancer (16, 20). The PEBP2/CBF site is present in the Tα4 element of the TCRα enhancer, Tβ3 and Tβ4 elements in the TCRβ enhancer, the δE3 element of the TCRδ enhancer, and the NF-γ1, NF-γ3, and NF-γ4 elements of the TCRγ enhancer (22, 36). PEBP2/CBF- and GATA3-binding sites are not always concurrently present in the same core elements in the TCR enhancers. Both PEBP2/CBF and GATA3 may contribute to the T-cell-specific transcriptional regulation by interacting with other factors.

The several species of the m*AML1*/*PEBP2*α*B* transcripts (7.9, 6.2, 3.8, and 2.1 kb) found in the thymus appeared to be similar in size to those found in several mouse hematopoietic cell lines, including T- and human myeloid leukemia cell lines, as well as bone marrow cells (2, 26). We observed that the 7.9- and 3.8-kb transcripts present in fibroblasts exclusively encode PEBP2αB2(mAML1-387) and αB1(mAML1-451) proteins, respectively (1). The αB1(mAML1-451) and αB2(mAML1-387) proteins are isoforms and show a distinct DNA-binding ability and transcriptional activation, suggesting that relative amounts of the two proteins in a given cell are important to determine the level of transcription (1, 38). Considering the fact that several species of the transcripts were detected in the thymus, it is possible that a more complex interaction among multiple isoforms of the mAML1/αB protein takes place in T cells. It is important, therefore, to examine whether the 6.2- and 2.1-kb transcripts that were detected in the thymus but not in fibroblasts can encode distinct polypeptides other than the reported αB1(mAML1-451) and αB2(mAML1-387) proteins.

The origin and the differentiation potential of T-cell precursors are different in early fetal life and adulthood (hematopoietic liver in the fetus and bone marrow in adults) (14). Transcripts of both the α*A* and m*AML1*/α*B* genes were detected in the thymus from day 16 embryos and in CD4$^-$ CD8$^-$ thymocytes from the adult thymus, implying the relatively early onset of the expression of α*A* and m*AML1*/α*B* during T-cell matu-

FIG. 8. (A) Detection of the transcripts of m*AML1*/*PEBP2*α*B* in a day 12 fetal liver by means of in situ hybridization. (B) Hematoxylin staining of the same section.

FIG. 9. (A) Detection of the transcripts of m*AML1*/*PEBP2αB* in bone marrow cells. (B) Hematoxylin staining of the same section.

ration. The T cells expressing γ/δ TCRs appear at earlier stages of T-cell development than the α/β cells (13, 15). Although the PEBP2/CBF site in the enhancer of TCRδ plays an essential role in the TCRδ enhancer activity in Jurkat T cells (10), the data presented here did not directly show whether the γ/δ cells express *αA* and m*AML1*/*αB*. It will be of interest to determine whether PEBP2/CBF plays some roles in T-cell development from the stem cells of both fetal liver and bone marrow origins.

The expression of m*AML1*/*αB* is not limited to cells of T-cell lineage, since its transcripts were detected in spleens from nude mice which are devoid of T cells. In fact the m*AML1*/*αB* transcripts were detected in hematopoietic cells in the day 12 fetal liver. Although the lineage of signal-positive cells was not identified, they represented a significant number in the section examined. Since about 80% of hematopoietic cells present in the fetal liver are considered to be erythroblasts (14), it is likely that erythroblasts express m*AML1*/*αB*, although the possibility cannot be excluded that the cells in other lineages also express the gene. We also detected positive signals for the m*AML1*/*αB* transcripts in some bone marrow cells from newborn mice by in situ hybridization. As mentioned above, *AML1* is located at the breakpoints of t(8;21) and t(3;21) associated with AML (4, 25–27, 29). In bone marrow, m*AML1*/*αB* was expressed in rapidly growing cells at the blast stage, probably myeloblasts and/or erythroblasts, but it appeared to be down-regulated in mature granulocytes. This finding suggests that the proto-oncogene *AML1* is expressed at some specific stage during the myeloid lineage differentiation. The chimeric protein AML1/MTG8(ETO), which is generated as a result of t(8;21) and is expressed in leukemic cells carrying t(8;21), contributes to the maintenance of the proliferation potential of the leukemic cells apparently by inhibiting their differentiation (34). An attractive model would be that a chimeric protein exerts the above effects by interfering with the function of AML1 protein.

More detailed analyses of the *AML1*/*αB* gene expression along with hematopoietic cell differentiation will be necessary for a more precise understanding of the mechanism of myeloid leukemogenesis.

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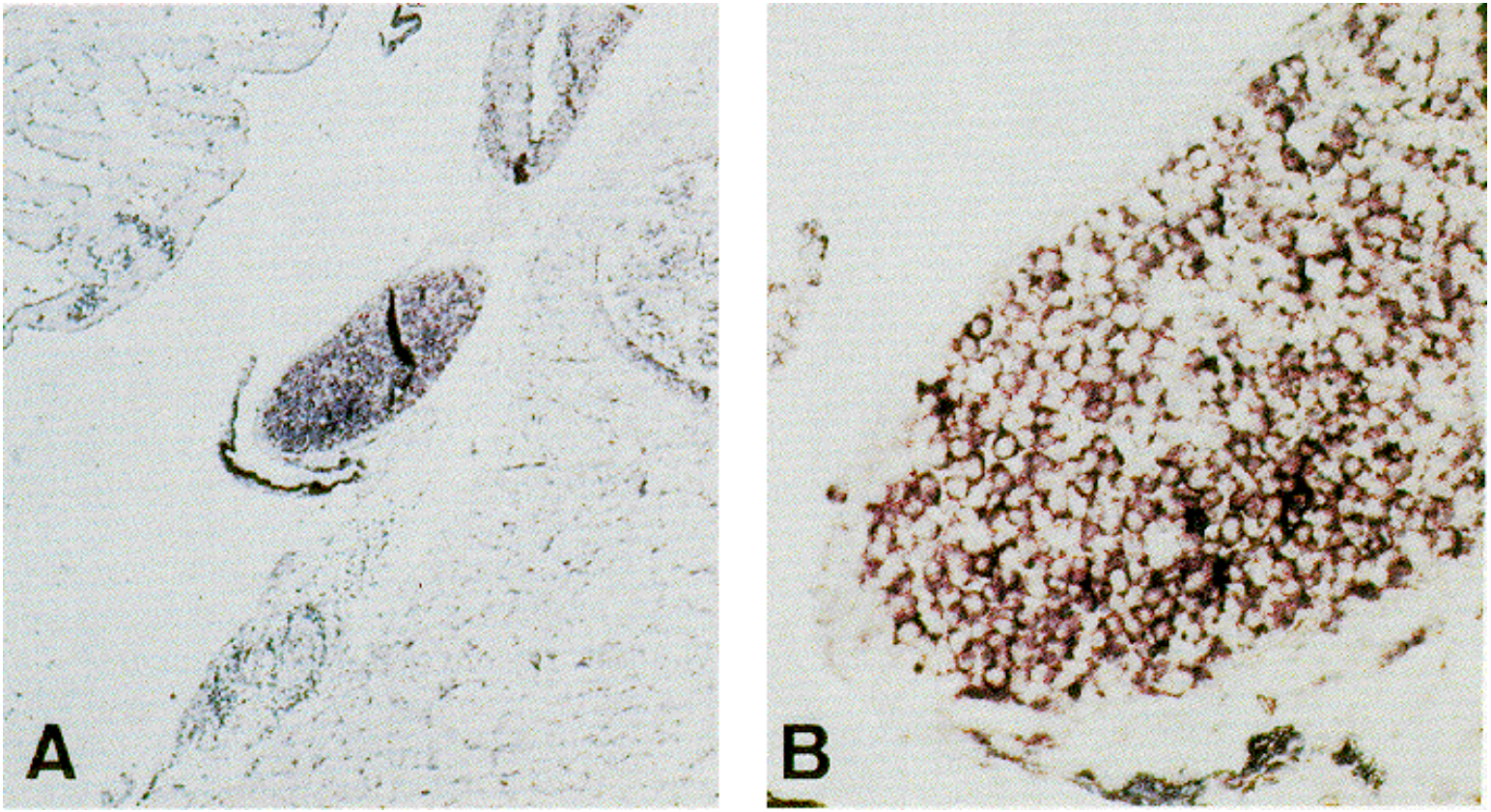
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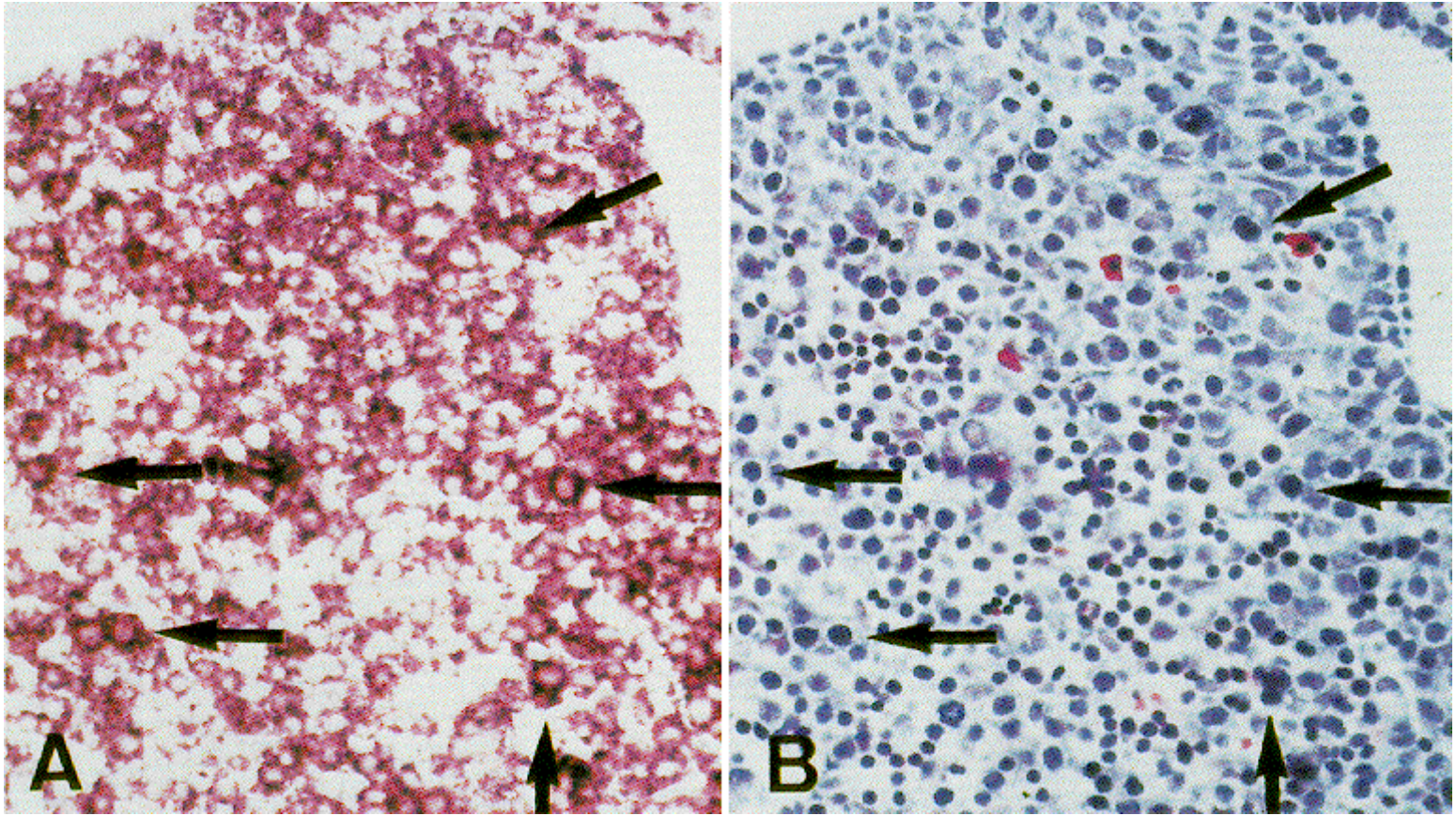
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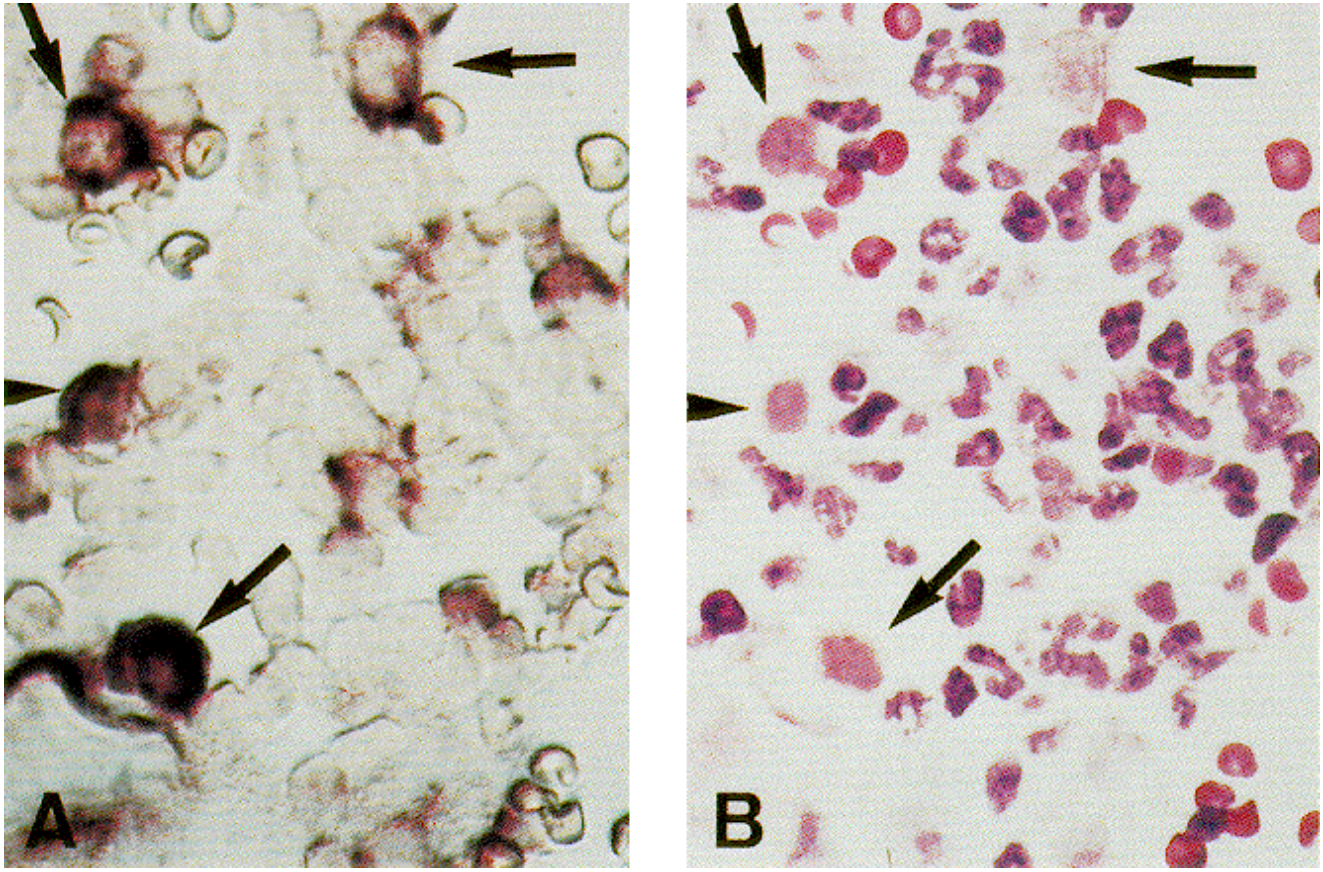
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