Isolation of a cDNA copy of an RNA species expressed in murine pre-B cells

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A part of a gene, pZ 183, was isolated from a cDNA library constructed from poly(A)-containing RNA of the chemically induced murine pre-B lymphoma 70Z/3. This DNA sequence was found to be present in one of 5000 cDNA clones in the unsubtracted library, and in one of 350 cDNA clones in a library in which sequences common to 70Z/3 and to a T cell hybridoma had been subtracted. This indicates that the corresponding gene belongs to the genes which are expressed with low to medium abundance in the pre-B cell line 70Z/3. The gene was found to be expressed in pre-B lymphomas, but not in B lymphomas, myelomas, T cell lymphomas, macrophage tumors or a fibroblast line. By *in situ* hybridization it was detected in 2–4% of fetal liver cells between day 15 and 16 of gestation, and in 1% of adult bone marrow cells. It was not detected in thymus cells, nor in mature T cells, spleen, adult liver, kidney, heart, lung or brain. The size of the transcript found in all pre-B cell lymphomas is 1.2 kb in length. The pZ 183 DNA sequence would appear to be a useful B-lineage specific marker which identifies pre-B cells at various stages before the mature B cell.

Key words: B cell-specific cDNA library/*in situ* hybridization/pre-B cell

Introduction

B lymphocytes develop from pluripotent stem cells in a series of differentiation steps in specialized organs such as fetal liver and bone marrow. During this development immunoglobulin (Ig) gene segments are rearranged to form complete Ig genes for heavy (H) and light (L) chains (Tonegawa, 1983). Rearrangements appear ordered in time of development: H chain gene segments are rearranged before L chain gene segments (Maki *et al.*, 1980; Alt *et al.*, 1981; Perry *et al.*, 1981; Coffmann and Weissman, 1983; Reth *et al.*, 1985). This distinguishes different stages of precursor (pre) B cell development. The most immature pre-B cell appears to have only D_H segments rearranged to J_H segments. Pre-B cells follow in which V_H segments have been rearranged to D_HJ_H segments (Alt *et al.*, 1981, 1984; Sugiyama *et al.*, 1983). The next stage is a pre-B cell which has not only rearranged V_HD_HJ_H, but also V_K to J_K (Maki *et al.*, 1980; Lewis *et al.*, 1983; Burrows *et al.*, 1983), and finally, V_λ to J_λ (Hieter *et al.*, 1981). The result of this process of B cell development is a resting, IgM- and/or IgD-positive B cell which can be stimulated by antigen or mitogen to divide and to mature into memory cells and into Ig-secreting plasma cells which often have switched their IgH gene expression and therefore synthesize other classes of Ig.

The identification of stages of pre-B and B cell development is also aided by monoclonal antibodies which characterize surface membrane markers that are preferentially expressed on subsets

of pre-B and B lymphocytes or plasma cells (McKearn *et al.*, 1984; Coffmann and Weissman, 1981; Kincade *et al.*, 1981). In many cases, however, these markers are also expressed on other lineages of blood cells.

There is, therefore, a need to find genes and gene products additional to Ig chains which characterize a given stage of B cell differentiation from stem cells. Abelson virus (A-MuLV) transformed pre-B cell lines have been obtained from murine fetal liver and adult bone marrow and provide model cell lines from which the molecular and cellular events of pre-B cell development can be followed (Reth *et al.*, 1985; Alt *et al.*, 1981). The B cell-specific genes could help to identify the sites of normal pre-B cell generation in the body, to follow the kinetics of normal pre-B cell development and to characterize the progeny of a pre-B cell. The genes could help to isolate normal pre-B cells for the study of regulatory processes and the molecular mechanisms of pre-B cell growth control and of Ig gene rearrangement in pre-B cells.

Thus we have constructed a library of cDNA clones, isolated from the pre-B cell line 70Z/3 subtracted by a pool of mRNA molecules from a T cell hybridoma, to search for pre-B cell-specific DNA sequences and genes. Here we report the identification of a cDNA clone, the gene of which is expressed at several stages of pre-B cell development but not in B cells, plasma cells or cells of other lineages.

Results

Construction of a cDNA library enriched for pre-B cell-specific sequences, and isolation of a pre-B cell-specific DNA sequence

To isolate pre-B cell-specific cDNA and construct phage clones from it, microsomal poly(A)⁺ RNA was isolated from the cell line of the chemically induced pre-B cell tumor 70Z/3 (Paige *et al.*, 1978). This cell line has been shown to express μ -heavy (H) chain, but no light (L) chains, and therefore was identified as a surface Ig-negative pre-B cell (Paige *et al.*, 1978; Sakaguchi *et al.*, 1980; Perry and Kelley, 1979). A cDNA library was constructed from this RNA as described in detail in Materials and methods. DNA sequences common to 70Z/3 and to a T cell hybridoma, K62, obtained in a fusion of the thymic lymphoma BW 5147 with an alloreactive, H-2^k-specific helper T cell clone, were removed from this cDNA library by hybridization, described in detail in Materials and methods. The non-hybridized fraction of cDNA molecules was then used to construct a phage library.

For a first screening this selected cDNA library was tested (i) with radioactive pre-B cDNA and (ii) with radioactive K62 T cell hybridoma cDNA. The frequency of pre-B cell-specific cDNA clones in the T cell cDNA-subtracted library was found to be 14 in 5000, while there were 10 in 50 000 in the unsubtracted, original 70Z/3 pre-B cDNA library. Two hundred plaques positive with only the pre-B cDNA but not with the K62 T cell cDNA were selected and further processed.

In the second screening these clones were then individually grown up, the phage DNA containing pre-B-specific sequences was extracted, the *Eco*RI insert fragments were radiolabeled by

Table I. Cell lines tested for the expression of pZ 183 transcripts

Differentiation type	Name of clone	Transformation (induction)	Tissue source	Strain	IgH-IgL genes protein		IgH-IgL chain	Phenotype ^c			
								B220	sIgM	AA4	GF1
Pre-B cell	40E-1	A-MuLV ^a	d 13-14 FL ^b	BALB/c	R ^c	E ^d	— —	+	—	+	+
	220-8	A-MuLV	Adult BM	BALB/c	R	E	— —	+	—	+	+
	204-1-8	A-MuLV	Adult BM	BALB/c	R	E	— —	+	—	+	—
	298-18	A-MuLV	Adult BM	BALB/c	R	E	— —	+	—	+	—
	230-238	A-MuLV	Adult BM	C57B1/6	R	E	— —	+	—	+	+
	28C-9	A-MuLV	d 17-19 FL	BALB/c	R	E	+	+	—	+	+
	204-3-1	A-MuLV	Adult BM	BALB/c	R	E	+	+	—	+	+
	18-81	A-MuLV	Adult BM	BALB/c	R	R	+	+	—	+	—
	70Z/3	Nitrosourea		(C57B1/6 × DBA/2)F ₁	R	R	+	+	—	+	+
Mature B cell	WEHI-279	Radiation		NZC				+	sIgM sIgD	+	+
	WEHI-231	Mineral oil		(BALB/c × NZB)F ₁				+	sIgM	—	—
	A20-3	Spontaneous		BALB/c				N.T. ^f	sIg	N.T.	N.T.
	M124.0	Spontaneous		BALB/c				N.T.	sIg	N.T.	N.T.
	K46.R.18	Spontaneous		BALB/c				+	sIgM sIgD	N.T.	N.T.
	2PK-3	Mineral oil		BALB/c				—	sIgD	+	+
Plasma cell	MPC-11	Mineral oil		BALB/c				—	—	—	—
	SP2/0	Hybridoma		BALB/c				N.T.	N.T.	N.T.	N.T.
	X63	Mineral oil		BALB/c				N.T.	N.T.	N.T.	N.T.
	J558	Mineral oil		BALB/c				—	—	—	—
T cell	EL4/9			BALB/c				N.T.	N.T.	N.T.	N.T.
	BW 5147	Spontaneous		AKR/J				N.T.	—	—	—
	K62	Hybridoma		AKR/J × C57B1/6				N.T.	N.T.	N.T.	N.T.
Other lineage cell	p388D1			DBA/2							
	WEHI-3			BALB/c							
	Ltk ⁻			C3H							

^aA-MuLV, Abelson murine leukemia virus.^bDay 13–14 fetal liver.^cR=rearranged in immunoglobulin genes.^dE=embryonic configuration in immunoglobulin genes.^eReferred from McKearn and Rosenberg (1985).^fN.T., not tested.

extension with random primers (Feinberg and Vogelstein, 1984) and used for hybridization with a panel of RNA preparations from different cell lines of different differentiation lineages at different stages of differentiation.

From two hundred 70Z/3-specific cDNA clones, clone pZ 183 was selected for further studies. The sequence was found to be expressed 1.5 times more than μ -heavy chain-specific mRNA in the 70Z/3 tumor cells. This level of expression is in the range of medium abundance and thus is estimated to represent 100–300 mRNA copies per cell (Hastie and Bishop, 1976).

Expression of the pre-B cell-associated pZ 183 sequences in transformed cell lines of different lineages and stages of differentiation
RNA extracted from a variety of murine cell lines with B cell characteristics, i.e. of pre-B, B and plasma cell phenotypes, as well as from cells of the T cell and monocyte lineage (listed in Table I) were tested for expression of pZ 183 sequences. The cell lines are considered to be 'frozen' at a certain stage of normal cell development and thus are thought to represent the phenotype of these normal counterparts. They are, therefore, useful since they provide large amounts of homogenous cells as sources of genes expressed in a lineage- and differentiation-specific manner.

The results in Figure 1 show that pZ 183 sequences are transcribed preferentially in the cell lines of the precursor B cell stages, including $c\mu^+$, sIg⁻ pre-B, 28C-9, 204-3-1, 18-81, 70Z/3 as well as $c\mu^-$, sIg⁻ progenitor cells, 40E-1, 220-8, 204-1-8, 230-238. All of such cells tested proved to be pZ 183

positive (Figure 1 and Table I). On the other hand pZ 183 transcripts are not detected within the limits of detection of our assays in early mature B cells (sIgM⁺) (WEHI-231), intermediate mature B cells (sIgM⁺, sIgD⁺) (WEHI-279), pre-secreting B cells (sIgD⁺), (2PK-3) or plasma blasts (SP2/0, MPC-11, X-63, J558). pZ 183 transcripts are also not detected in cells of T lineages, BW5147, EL4, K62, in monocytes, P388D1, WEHI-3 and in a fibroblast cell line, Ltk⁻.

These data further suggest that pZ 183 sequences are only transcribed at the pre-B cell stage of B cell differentiation. In this pattern of differentiation expression of pZ 183 is similar, but not identical to the expression of the surface antigen detected by the monoclonal antibody AA4 (McKearn *et al.*, 1984). The exceptions to this rule are the cell lines 2PK-3 and P388D1 which are pZ 183 negative, but AA4 positive.

Expression of pZ 183 sequences in pre-B cells at various stages of Ig-gene rearrangements

A set of Abelson-virus (A-MuLV) transformed pre-B cell lines exists that is 'frozen' at different stages of Ig gene rearrangements (Alt *et al.*, 1984; Yancopoulos *et al.*, 1984). Differential expression of pre-B cell-associated surface antigens characterized by the monoclonal antibodies 14.8, AA4 and GF1 has been observed with these cell lines (McKearn and Rosenberg, 1985). B cell precursors with fully rearranged ($V_H \rightarrow D_H \rightarrow J_H$) or with incompletely rearranged ($D_H \rightarrow J_H$) IgH genes both express in general the B 220 surface glycoprotein and the antigen recognized

Fig. 1. Expression of pZ 183 transcripts in the cell lines in different stages and lineages. Total cytoplasmic RNA (30 μ g) from various cell lines was electrophoresed on 1.2% agarose/formaldehyde gel, transferred to nitrocellulose filter and hybridized with the radioactive probe of pZ 183 (a) or β -actin (b). After hybridization under the conditions described in Materials and methods, the filters were finally washed with $0.2 \times$ SSC, 1% SDS at 65°C and exposed to film in the presence of an intensifying screen at -70°C for 72 h.

by the monoclonal antibody AA4, whereas some of these cells do not express an antigen recognized by the monoclonal antibody GF1. Therefore, pZ 183 expression was probed with RNA extracted from A-MuLV-transformed pre-B cell clones obtained from different strains of mice, from different organs at different times of gestation, characterized by different stages of rearrangements of IgH and L chain genes and by other surface markers (listed in Table I).

Results in Figure 1 show that pZ 183 is expressed in all pre-B cell lines, as they originate either from pre-B cells of day 13–14 (40E-1), day 17–19 of gestation in fetal liver (28C-9), and from adult bone marrow (220-8, 204-1-8, 230-238, 204-3-1, 18-81). It is, of course, also expressed in the 70Z/3 pre-B cell line obtained by chemical carcinogenesis from which the cDNA library was made. pZ 183 is expressed in lines which have only $D_H \rightarrow J_H$ rearranged, clones which have $V_H \rightarrow D_H \rightarrow J_H$ rearranged (28C-9, 220-8, 18-81, 70Z/3, 204-1-8, 230-238), clones which either express ($c\mu^+$) (28C-9, 204-3-1, 18-81, 70Z/3) or do not express ($c\mu^-$) μ heavy chain proteins (40E-1, 220-8, 204-1-8,

230-238), and clones which either express (40E-1, 220-8, 230-238, 28C-9, 204-3-1, 70Z/3) or do not express the GF1 surface antigen (204-18, 18-81).

The level of expression of pZ 183-specific transcripts was estimated by RNA dot blot analysis of serially diluted RNA samples from different cell lines (Figure 2). If the B cells, plasma cells and cells of other lineages express any pZ 183-specific transcripts at all, their level of expression must be 10–50 times lower than that found in the pre-B cell lines. In all pre-B cell lines tested the level of expression of pZ 183-specific transcripts was comparably higher (Figures 1 and 2).

The size of the pZ 183-specific transcript(s) was analyzed in RNA prepared from either unstimulated or LPS-stimulated 70Z/3 cells by Northern blot analysis (Figure 3). pZ 183-specific RNA from both types of cells was found to be 1.2 kb in size. LPS stimulation of 70Z/3 cells which induces further maturation of these cells so that they express kL chains (Paige *et al.*, 1978; Sakaguchi *et al.*, 1980) does not influence the level of transcription or the size of the pZ 183-specific transcript. In all other pre-B

Fig. 2. Quantitative dot blot analysis of pZ 183 transcripts in the cell lines. Serial dilutions of total cytoplasmic RNA from various cell lines were dotted onto nitrocellulose filter for hybridization with the probe of pZ 183 (a) or β -actin (b). Prehybridization, hybridization and washing conditions are the same as in Figure 1.

cell lines the size of the pZ 183-specific transcripts was found to be the same, i.e. 1.2 kb (Figures 1 and 3).

Expression of pZ 183 sequences in different lymphoid tissues

In a first analysis RNA was prepared from cells of spleen, thymus, kidney, bone marrow, lung, heart, brain and liver. It is clear from the data presented in Figure 4 that this analysis yielded no positive signals from RNA of all these organs. This indicated that the relative contribution of pre-B cells expressing pZ 183 in all of these organs must be too low in the total mixture of all other cells to be detectable by Northern gel analysis.

We therefore resorted to an *in situ* hybridization technique developed for tissue sections and single cells of lymphoid organs (Berger, 1986a,b) to probe for expression of pZ 183 sequences in these organs. For this, an insert fragment of pZ 183 of 380 bp was introduced into M13 phage to obtain single-stranded pZ 183-

specific probe which could be used in radiolabeled form in the *in situ* hybridization technique. The pZ 183-specific sequence was radiolabeled by primer extension DNA synthesis and the labeled pZ 183 sequences, separated from the adjacent M13 sequences, were purified as the single strand probe molecules by gel electrophoresis.

As can be seen in Figure 5, the radiolabeled pZ 183 probe readily detected specifically hybridizable cells expressing pZ 183-specific RNA within 15 day fetal liver cells (Figure 5a), and in total adult bone marrow cells (Figure 5c), but not in small resting spleen cells (Figure 5d) (>1000 cells counted). As one control, 70Z/3 cells were found to be positive (data not shown). As another control the positive strand probe, as expected, did not label any cells of the fetal liver (Figure 5b) (>1000 cells counted). In fetal liver cells from day 15 of gestation, 2.5% of all cells (13 of 485 counted cells) were found to be pZ 183-

Fig. 3. Northern blot analysis of pZ 183 transcripts in RNA isolated from unstimulated and LPS stimulated 70Z/3. Poly(A)⁺ RNA (5 µg per lane) was isolated from 70Z/3 cells cultured with (b) or without (a) LPS (10 µg/ml) for 12 h. RNA electrophoresis, transfer to nitrocellulose filter, prehybridization, hybridization and washing conditions are as described in Figure 2. The blot was exposed to X-ray film for 24 h at -70°C in the presence of an intensifying screen. As size markers, denatured ³²P-radiolabeled λHindIII and φXHaeIII fragments were used.

positive while, at day 16, 4% (eight of 216 counted cells) of all cells were positive. In the adult bone marrow, 1% of cells (six of 619 counted cells) expressed pZ 183 transcripts.

Finally, the expression of pZ 183 sequences was studied in tissue sections on day 16 fetal liver. Figure 6 shows that the pZ 183-positive cells in fetal liver do not form foci, but are scattered in a diffuse way over large areas of the fetal liver.

It remains to be established by double, maybe even by triple marker analysis involving Ig as well as other pre-B cell stage specific markers, that the pZ 183-positive cells are, indeed, pre-B cells. If they are, their distribution in the fetal liver is similar to the distribution of pre-B cells detected as intracytoplasmically µ-heavy chain positive cells (Kamps and Cooper, 1982).

Discussion

The cDNA clone pZ 183 isolated from mRNA of the chemically induced murine pre-B lymphoma 70Z/3 has been used to detect the expression of the corresponding gene at the RNA level in cells of various lineages at various points in their differentiation.

Fig. 4. Northern blot analysis of pZ 183 transcripts in the cells of normal organs and tissues. Poly(A)⁺ mRNA of thymus, spleen, bone marrow, kidney, liver, heart, lung and brain (5 µg each) and poly(A)⁺ RNA from 70Z/3 cells (2 µg) were electrophoresed, stained with ethidium bromide (b), transferred to nitrocellulose filter and hybridized to radioactive pZ 183 probe (a). Blots were exposed to X-ray film for 7 days at -70°C in the presence of an intensifying screen.

The gene is expressed in pre-B cell lines ranging from very early stages of differentiation, e.g. before IgH or L chain expression at the level of D_H-J_H rearrangement, to pre-B cells which have rearranged both H and L chain loci.

The level of expression of pZ 183 in pre-B cells can be estimated to be of low to medium abundance, i.e. in the range of 100 mRNA molecules per cell. This estimate is derived from the frequency of pZ 183 sequences within the cDNA library constructed from 70Z/3 mRNA, i.e. one in 5000, under the assumption that one pre-B cell contains 5×10^5 mRNA molecules and that every mRNA molecule is transcribed reversely into cDNA with equal efficiency. The intensity of staining of single pre-B cells by radiolabeled pZ 183 *in situ* (Figure 5), in comparison with the intensity of staining of resting B cells observed with radiolabeled µ-heavy chain specific DNA probes (Berger, 1986a) further indicates such a level of pZ 183 mRNA expression. pZ 183, therefore, is expressed at levels similar to those reported for Lyt-2 (Nakauchi *et al.*, 1985) and for Ig µ heavy chains in 70Z/3 pre-B cells (Kelley *et al.*, 1985). From the quantitative dot blot analysis of RNA extracted from different cells as shown

Fig. 5. Detection of pZ 183-specific mRNA molecules in single cells of lymphoid organs by *in situ* hybridization. Bars 10 μ m. (a) Day 15 fetal liver cells hybridized with pZ 183-negative strand probe, pronase digestion 66 μ g/ml, exposure time 62 days. (b) As in (a) but hybridized with pZ 183-positive strand probe. (c) Adult bone marrow cells hybridized with pZ 183-negative strand probe, pronase digestion 33 μ g/ml, exposure time 49 days. (d) Small resting spleen cells hybridized with pZ 183-negative strand probe, pronase digestion 11 μ g/ml, exposure time 49 days.

in Figure 2 we conclude that pZ 183-specific RNA sequences must be at least 10–50 times less frequent in mature B cells, plasma cells, T cells and cells of other lineages than they are in pre-B cells, if they are expressed at all. We therefore cannot yet rule out that pZ 183-specific RNA exists in low abundance, i.e. between one and 10 molecules, in other cells of the B lineage or in other cell lineages.

In later stages of pre-B cell lines, represented by 70Z/3 cells stimulated by LPS, the onset of k-L chain expression within 6–12 h has been found (Sakaguchi *et al.*, 1980). It is evident that this LPS stimulation does not shut down the expression of pZ 183 (Figure 2), indicating that expression of L chains does not necessarily shut down expression of the pZ 183.

On the other hand, mature B cells expressing H and L chains were found within the limits of detection of our assays, i.e. with low to medium abundance, not to express pZ 183. This was true for early and late mature B cell lines expressing IgM only or IgM plus IgD, for plasma blastoid and plasma cell tumors as well as for normal B cells of spleen or bone marrow. Since pZ 183 expression was also undetectable in thymic lymphoma, T cell hybridomas, macrophage cell line, thymocytes, fibroblast cell line, liver cells, brain, lung, heart, kidney and in the majority of fetal liver or bone marrow cells, pZ 183 expression at low

to medium abundance levels can be regarded as highly restricted to pre-B cells. It is interesting to note that it is more restricted than the most restricted surface antigens found on pre-B cells, i.e. the AA4 and BP-1 antigens (McKearn *et al.*, 1984; Cooper *et al.*, 1986). The AA4 antigen, in contrast to pZ 183 expression, however, is also found on P388D1 macrophage cells and on 2PK-3 B lymphoma cells.

Thus, pZ 183 cannot code for the AA4 antigen. By the size of the transcript, it also cannot code for the BP-1 antigen which has been found to be a 140 kd glycoprotein. Again, by the size of the transcript, pZ 183 also does not code for either terminal transferase (Ladau *et al.*, 1984) which has 2.1 kb message and for MuLV-encoded proteins (Tidmarsh *et al.*, 1985), both of which are not really as restricted in their pattern of expression in different cell lineages as is pZ 183. The B cell lineage-specific antigen B220 (Ly-5), a 220 kd glycoprotein (Coffman and Weissman, 1981; Kincade *et al.*, 1981) which has an ~5.0 kb message (Shen *et al.*, 1985), also appears not to be a candidate for the pZ 183 phenotype since T200 is detected in most mature T cells while pZ 183 is not. B cell differentiation stage-specific expression of *N-myc* mRNA has been observed (Zimmerman *et al.*, 1986). While *N-myc* is expressed specifically in pre-B cells, *c-myc* is expressed in all cells of the B lineage which were tested.

Fig. 6. Localization of cells containing pZ 183-specific mRNA molecules in day 16 fetal liver. Cells were digested with 30 $\mu\text{g}/\text{ml}$ pronase. Exposure time 63 days. Bars 5 μm . (a) Hybridization with pZ 183-negative strand probe. (b) Hybridization with pZ 183-positive strand probe.

Therefore, N-myc expression while it is similar to that observed for pZ 183 in the B lineage, is also expressed as a 2.9 kb transcript in non-lymphoid cells and tissues such as brain, kidney, intestine, lung and heart, while pZ 183, 1.2 kb in size, is not. We are therefore still searching for a function of the pZ 183 gene product.

We have not yet found proof that the pZ 183-positive cells in bone marrow and fetal liver are, in fact, pre-B cells. This will require double staining for pre-B cell-specific markers such as for cytoplasmic μ heavy chain or the AA4, GF1 or BP-1 markers. If we assume for the moment that pZ 183-positive cells are, indeed, pre-B cells then the frequencies of pZ 183-positive cells in fetal liver at days 15 and 16 of gestation, determined here as 2–4%, appear somewhat higher than the frequencies of $c\mu^+$, $s\mu^-$ pre-B cells detected by fluorescein-labeled μ heavy chain-specific antibody staining, determined to be 0.06–0.13% at day 15 and 0.42–2% at day 17 of gestation (Raff *et al.*, 1976; Velardi and Cooper, 1974). It could, therefore, be possible that pZ 183 is expressed in pre-B cells of other genotype and phenotype, i.e. V_HDJ_H -rearranged, but $c\mu^- s\mu^-$ cells. Such $c\mu^- s\mu^-$ (V_HDJ_H) pre-B cells may include those that have made rearrangements with pseudo Ig-gene segments or are rearranged out of reading frame, i.e. are mistakes of the B cell differentiation process.

In contrast, the frequency of pZ 183-positive cells in bone marrow appears to be lower than that of $c\mu^+ s\mu^-$ pre-B cells, i.e. 1–2% versus 3.2% (Burrows *et al.*, 1978) or 3.4–4.9% (Velardi and Cooper, 1984), or 12% (Raff *et al.*, 1976; Rahal and Osmond, 1984). $c\mu^+ s\mu^-$ pZ 183⁺ cells of the B lineage may include those that have properly rearranged the H chain locus, but have made mistakes in light chain rearrangement, and therefore develop to mature, pZ 183-negative cells which, however, cannot express μ -heavy chains on the surface of the cells. The pZ 183 marker thus appears as an interesting analytical tool for a detailed study of B cell differentiation.

Materials and methods

Mice

(C57BL/6J $\times$ DBA/2J)F1 mice, 6–8 weeks old, and timed pregnancies of female C57BL/6J mice mated with DBA/2J mice induced by putting male and female mice together overnight were obtained from Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland. Day 1 of gestation was counted as the day after mating. Birth occurred on day 19.

Cells, cell lines and culture conditions

Fetal liver cells from timed pregnant mice were prepared as described (Melchers, 1977). Spleen cells, bone marrow cells and thymus cells were prepared as cell suspensions.

The pre-B cell Abelson lines, 40E-1, 220-8, 204-1-8, 298-18, 230-238, 28C-9, 204-3-1, 18-81 were obtained from Dr N. Rosenberg, Tufts University School, Boston, MA. 70Z/3 cells and WEHI-231 were obtained from Dr Christopher Paige from our Institute. The B cell lymphoma lines, WEHI-279 (Sibley *et al.*, 1980), 2PK-3 and the thymoma EL4/9 were given to us by Dr J. McKearn, Dupont Glenolden Laboratories, Glenolden, PA. The B cell tumor lines A20-3, M124.0, K46.R.18 were brought to our Institute by Dr J. Kim, National Institute of Health, Bethesda, MD. The macrophage lines P388D1 and WEHI-3 (D^-) were given to us by Dr N. Iscove (E.M.B.L. Heidelberg, FRG). The thymic lymphoma BW5147 (HPRT⁺, Oua⁺) was given to us by Dr Hyman, Salk Institute for Biological Studies, La Jolla, CA. The other cell lines were SP2/0 (given to us by Dr G. Köhler, Max Planck Institute for Immunobiology, Freiburg i. Br., FRG), MPC11 (given to us by Dr M. Scharff, Albert Einstein College of Medicine, Bronx, NY), and K62 T cell hybridoma line (given to us by Dr Antonius Rolink, Basel Institute for Immunology). All cell lines and their known properties are listed in Table I.

All cell lines were grown in RPMI-1640 medium containing 2 mM glutamine, 5% heat inactivated fetal calf serum (Gibco), β -mercaptoethanol (5×10^{-5} M), streptomycin (100 $\mu\text{g}/\text{ml}$) and penicillin (100 units/ml) in a 10% CO_2 atmosphere.

Microsome-bound polysomal RNA preparation

70Z/3 cells were grown to a density of 1×10^6 cells/ml and incubated for 15 min with cycloheximide (20 $\mu\text{g}/\text{ml}$) before harvesting. Microsome-bound polysomes were prepared as described (Mechler and Rabbitts, 1981). Briefly, cells were resuspended in 20 volumes of hypotonic buffer, 10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl_2 , 10 mM Vanadium Ribonucleoside Complex, 20 $\mu\text{g}/\text{ml}$ cycloheximide and allowed to swell for 15 min on ice. After homogenization by 30 strokes of a tight-fitting (B) Dounce glass homogenizer and centrifugation at 1000 g for 5 min, the cytoplasmic extract was diluted 5-fold in 2.5 M sucrose with TKM buffer (0.15 M KCl, 5 mM MgCl_2 , 50 mM Tris-HCl, pH 7.4) and layered over 2 volumes of 2.5 M sucrose TKM buffer. Two layers of sucrose TKM buffer were successively added, one with 2.05 M sucrose and a second with 1.3 M sucrose. The gradients were centrifuged for 5 h at 4°C in a SW27 rotor (Beckman Instruments, Inc.) at 25 000 r.p.m. Microsomes floated at the interface between the 2.05 and 1.3 M sucrose layers. Microsomes were dissolved in 0.5% NP-40 and layered over two layers of 2 M sucrose-TKM and 0.5 M sucrose-TKM (80 mM KCl, 5 mM MgCl_2 , 50 mM Tris-HCl, pH 7.4). After centrifugation at 35 000 r.p.m. for 4 h with a Beckman 60 Ti rotor, the polysomal pellet was dissolved in 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.2% SDS and then extracted with an equal volume of phenol:chloroform:isoamylalcohol (24:24:1).

Poly(A)⁺ RNA was prepared by repeated oligo(dT) cellulose chromatography (P-L Pharmacia, Uppsala, Sweden) in the presence of dimethyl sulfoxide as described (Bantle, 1976).

Construction of selected cDNA library

A subtracted cDNA library for B cell-specific clones was constructed essentially according to Davis *et al.* (1984). Briefly, the first cDNA strand was synthesized with 10 μg of microsome-bound polysomal poly(A)⁺ RNA from 70Z/3 in 50

mM Tris-HCl pH 8.3, 6 mM MgCl₂, 60 mM NaCl, 20 mM dithiothreitol (DTT), 10 µg/ml of oligo(dT) 12–18, 1 mM of each four deoxynucleotides, 100 µCi of [³²P]dCTP (~3000 Ci/mmol), 60 units/ml of placental ribonuclease inhibitor, 100 µg/ml of actinomycin D, and 100 units of AMV reverse transcriptase (Stehlin, Co., Basel, Switzerland) at 40°C for 2 h. After RNA hydrolysis, hybridization reactions were performed in 0.5 M phosphate buffer, 5 mM EDTA, 0.1% lithium laurylsulfate with twenty times excess amount of polysomal poly(A)⁺ RNA from T cell hybridoma K62 at 68°C for 16–20 h to achieve a Cot of 2000. The single-stranded fraction after hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) chromatography in 0.12 M phosphate buffer, 0.1% lithium laurylsulfate, 5 mM EDTA at 65°C was re-subtracted in the same conditions as above. The enriched single-stranded cDNA obtained by these procedures (1.75% of the starting amount of cDNA) was made double stranded by the Klenow reaction in 50 mM Hepes-KOH pH 6.9, 70 mM KCl, 20 mM MgCl₂, 10 mM DTT, 50 µg/ml bovine serum albumin, 0.5 mM of each deoxynucleotides, and 300 units/ml of Klenow fragment of DNA polymerase I (Stehlin) at 12°C for 20 h and followed by the reverse transcriptase reaction in 0.1 M Tris-HCl pH 8.3, 140 mM KCl, 10 mM MgCl₂, 30 mM β-mercaptoethanol, 1 mM of each of the four deoxynucleotides and 800 units of AMV reverse transcriptase at 42°C for 1 h.

After removal of the hairpin loop with S1 nuclease (P-L Pharmacia), the cDNA was methylated with *Eco*RI methylase (New England Bio-Labs) and the ends were filled in with T4 polymerase (P-L Pharmacia). *Eco*RI linkers (New England Bio-Labs) were ligated to both ends by the T4 ligase (Stehlin) reaction with 15% polyethyleneglycol 6000 at 12°C for 16 h and were digested with *Eco*RI. After the digestion, the cDNA preparation was purified from the digestion product by passage through a column of Bio-Gel A15M, 100–200 mesh (Bio-Rad). Samples (~30 ng) were ligated to λgt11 DNA (3 µg) that had been cut with *Eco*RI and treated with calf intestinal phosphatase. Ligated samples were packaged into phage (Young and Davis, 1983) and plated onto *Escherichia coli* RY 1088. Approximately 5 × 10⁴ total recombinants were obtained.

Differential hybridization of recombinant clones with radioactive B cell and T cell cDNAs

Recombinant phage clones were plated onto YT plates with ampicillin (25 µg/ml) and transferred to nitrocellulose filter as described (Benton and Davis, 1977). The duplicate filters were screened with radioactive cDNA probes from the mRNA of either 70Z/3 or K62 cells. Radioactive cDNA probes were synthesized from each mRNA preparation by the AMV reverse transcriptase reaction with the random primer of oligodeoxynucleotide mixtures produced from calf thymus DNA (P-L Pharmacia).

Pre-hybridization and hybridization were performed in 5 × SSPE (750 mM NaCl, 50 mM NaH₂PO₄, 5 mM EDTA), 5 × Denhardt's solution, 1% SDS, 1 µg/ml tRNA from *E. coli*, 1 µg/ml poly(A), 100 µg/ml of denatured salmon sperm DNA at 68°C and the filters were washed finally with 0.1 × SSC, 1% SDS at 65°C. The clones which showed positive after 7 days of exposure with intensifying screen at –70°C were rescreened with both probes and 200 individual phage clones were selected by this procedure out of 50 000 clones.

Radiolabeling of insert DNA fragments

Extraction of individual phage clone DNA was done by a method described by Yamamoto *et al.* (1970). *Eco*RI-digested insert fragments from phage clone DNA were separated by electrophoresis through a 1% low melting point agarose gel, and radiolabeled with [³²P]dATP by the Klenow fragment reaction with the random hexanucleotide primers from calf thymus DNA (Feinberg and Vogelstein, 1984). β actin probe was radiolabeled as well with the *Pst*I fragment of plasmid pAL41, which was provided by Dr M.Buckingham (Pasteur Institute, France).

RNA dot blot and Northern blot analysis

Preparation of cytoplasmic RNA for RNA dot blot analysis and transferring to nitrocellulose filters were done as described (White and Bancroft, 1982). For the Northern blot analysis, cytoplasmic RNA was prepared by the guanidium thiocyanate/cesium chloride buoyant density centrifugation method (Chirgwin *et al.*, 1979). Some of the RNA samples were further purified by oligo(dT) cellulose chromatography.

One to forty micrograms of RNA sample were electrophoresed by the agarose/formaldehyde method and transferred to nitrocellulose filters as described (Maniatis *et al.*, 1982).

Prehybridization and hybridization were performed as described in the sections on differential hybridization but with 50% formamide at 42°C. The filters were finally washed with 0.2 × SSC, 1% SDS at 65°C.

In situ hybridization with single cells or organ sections

Preparation of ³H-labeled single-strand probe and the *in situ* hybridization technique were as described (Berger, 1986a,b).

Briefly, the pZ 183 fragment (380 bp) was inserted into the M13 mp11 *Eco*RI site, the construct grown in bacteria and the single-strand phage DNA extracted from these bacteria. The positive strand phage DNA and the opposite strand phage DNA were tested by RNA dot hybridization on RNA samples of 70Z/3.

After the Klenow fragment reaction of template DNA with 17-mer sequencing

primer in the presence of the four ³H-labeled nucleotides, the mixture was digested with *Bam*HI and the 0.4-kb DNA fragment on the polyacrylamide gel (6%) was electroeluted.

Ten thousand cells were attached to the glass slide, fixed, treated with pronase, prehybridized and hybridized with 10 µl of single strand radiolabeled DNA probe (6500 d.p.m./µl) in prehybridization solution (50% formamide, 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, 1 × Denhardt's solution, 150 µg/ml sonicated salmon sperm DNA, 500 µg/ml tRNA) for 3 days at 35°C. The slides were washed within 24 h with three to four changes of 50% formamide, 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5. Photographic development of the slides and counting of the grains were done as described (Berger, 1986a).

Fetal liver organs were snap frozen in liquid nitrogen and prepared as 5 µm thick sections on glass slides. The *in situ* hybridization was carried out as described above.

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