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Dynamic Chromatin Remodeling in the Vicinity of J Chain Gene for the Regulation of Two Stage-specific Genes during B Cell Differentiation

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Dynamic chromatin remodeling during B cell differentiation was identified in the vicinity of J chain gene. In pre-B cells, the enhancer-containing DNase I hypersensitive sites (HSSs) 3–4 were open. However, these HSSs 3–4 turned out to be unassociated with J chain gene expression, as the J chain promoter-containing HSS1 remained in a closed state. The open enhancer HSSs 3–4 in the pre-B cells might be related to the expression of a pre-B cell-specific gene upstream of the HSSs 3–4, which was identified in our Northern blot analyses. The HSSs 3–4 are then closed in the next immature and mature B cell stages until the IL-2 opens the HSSs 3–4 again as well as HSS1 to express J chain gene in the primary immune responses. The dynamic regulation of chromatin structure during B cell differentiation for the expression of two stage-specific genes will provide a good model system for the study of B cell differentiation and gene expression.

Keywords: B Cell Differentiation; Chromatin; DNase I Hypersensitive Site; J Chain Enhancer.

Introduction

Eukaryotic genome is maintained by packaging its large DNA into chromatin, which is grossly divided into two domains of euchromatin and heterochromatin. Generally, the deeply staining heterochromatin is in a higher order chromosomal package and inaccessible, whereas the diffuse euchromatin appears to be accessible and active (Weiler and Wakimoto, 1995). However, the actual accessibility and activity in the chromatin domains should be assessed at the molecular level. DNase I hypersensitivity assay has successfully been used to assess the chromatin structure of many genes (Gross and Garrard, 1988). DNase I hypersensitive sites (HSSs) are often related to many DNA regulatory elements such as locus control region (LCR), matrix attachment region (MAR), boundary element/insulator, enhancer, or promoter.

The major stages of B cell development include pro-B, pre-B, immature B, mature B, and plasma cells. Our previous study demonstrated that the expression of J chain gene is strictly restricted to the antigen-stimulated plasma cell stage (Koshland, 1985). This limited expression of J chain gene was shown to be regulated by the chromatin accessibility (Kang *et al*., 1998). There has been seven HSSs upstream of the immunoglobulin J chain gene, of which HSS1 is its promoter and HSSs 3–4 contain its enhancer activity. The function of HSSs 5–7 was not defined, although HSSs 5–6 correlated with the J chain gene expression and HSS7 was shown to be open irrespective of its gene expression.

We extended the analysis of these hypersensitive sites further into the cell lines representing the early B cell development to see if the HSSs correlated with the J chain gene expression. Unexpectedly, we found a surprising phenomenon of chromatin accessibility in pre-B cells such as PD31 or PD36 cell lines. In these pre-B cells, the enhancer-containing HSSs 3–4 were open although the J chain promoter-containing HSS1 was shown to remain in a closed state. The presence of HSSs 3–4 in the absence of HSS1 in a pre-B cell stage led us to search for a potential target gene of HSSs 3–4. The pre-B cell-specific expression of a gene was indeed identified by performing a northern blot experiment with a genomic DNA fragment upstream of HSSs 3–4, indicating a potential usage of the

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Abbreviations: DMS, dimethyl sulfate; HSS, DNase I hypersensitive site.

open enhancer HSSs 3–4 for its expression. The open HSSs 3–4 are then closed in the next immature and mature B cell stages, and open again with the J chain promoter containing HSS1 in the plasma cell stage for J chain gene expression (Kang *et al*., 1998). To our knowledge, this is the first demonstration of a dynamic open-closed-open regulation of chromatin structure during B cell differentiation.

Materials and Methods

Cell culture The cell lines were maintained at 37°C in DMEM or RPMI 1640 (Gibco BRL, Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 1 mM MEM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin in an atmosphere of 7% CO_2 saturated with water. All the cell lines used in this paper originated from mice.

DNase I hypersensitivity assay Cells in logarithmic-phase growth ($0.5–1.0 \times 10^6$/ml, 85 ml) were harvested by centrifugation and then allowed to swell in 10 ml of hypotonic solution [10 mM Tris·HCl (pH 7.9), 10 mM KCl, 3 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 0.75 mM spermidine, 0.15 mM spermine] for 20 min on ice. Triton X-100 was added to a final concentration of 0.1%, and the cell membranes were broken by 20 strokes with a B pestle in a Dounce homogenizer. After the homogenate was centrifuged at 4°C for 7 min at $500 \times g$, the pelleted nuclei were resuspended in 2.5 ml of DNase I digestion buffer [0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM dithiothreitol, 0.1 mM EGTA, 5% glycerol]. For the zero time point in the assay (DNase I untreated control), 500 μl of the nuclear suspension was removed and added to 50 μl of quenching solution (200 mM EDTA, 10% SDS). Five units of DNase I (1 U/μl, 0.5 μg/μl) were then added to the remaining 2 ml. The suspension was then mixed well at room temperature and digestion was stopped at the time points of 0.5, 1, 2, and 3 min by transferring 500 μl samples to 50 μl of quenching solution. The quenched viscous DNA samples were treated with 20 μl of RNase A (10 mg/ml) for 30 min at 37°C and then with 18 μl of proteinase K (20 mg/ml) overnight at 55°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation, digested with appropriate restriction enzymes, and analyzed in Southern blots. Southern blots (Zeta-Probe, Bio-Rad) were performed with random primer-^{32}P-labeled probes.

DMS *in vivo* footprinting via ligation-mediated (LM) PCR DMS *in vivo* footprinting analysis by ligation-mediated (LM) PCR was performed as described (Ausubel *et al*., 1994) with the following modifications. For the preparation of the DNA, 70 ml of the cells in logarithmic-phase growth ($0.5–1.0 \times 10^6$ cells/ml) were pelleted and resuspended in 2 ml of the same media used for cell culture. The resuspended cells were mixed with 20 μl of 10% DMS (in ethanol) and incubated at room temperature for 2–5 min. The reaction was stopped by adding 3 ml of 2-mercaptoethanol (1 M) and then 20 ml cold PBS. After the cells were collected by centrifugation in the cold, they were washed once with 40 ml of cold PBS, resuspended in 2 ml of cold PBS, and lysed by adding 250 μl of quenching solution (200 mM EDTA, 10% SDS, 50 mM EGTA). The lysed cells were treated with 100 μl of RNase A (10 mg/ml) for 30 min at 37°C and then with 90 μl of proteinase K (20 mg/ml) overnight at 55°C. The DNA was purified and treated with 1 M piperidine as described by Ausubel *et al*. (1994).

LM-PCR was performed using 2.5–5 μg samples of the purified DNA. The primers for the noncoding strand were as follows: NP1 for first-strand synthesis, CCCAATGAAGTTTGGAAGTAGACCAC; NP2 for amplification of ligated DNA, AAGTAGACCACTTGTCGATTAGGACC; and NP3 for labeling, TTGTCGATTAGGACCTGTGGGTAATGG. The equivalent primers for the coding strand were: CP1, GGGTATCAGACAGCAAAATCTTAAATC; CP2, TCTTAAATCTAAAAAGAACTCAAAACATAG; and CP3, AAAGAACTCAAAACATAGGGAGAAACC. Vent(exo$^-$) DNA polymerase (New England Biolabs) was used in the PCR with the supplied Thermopol buffer so that it was necessary to calculate the melting temperature for each primer using the formula given by Ausubel *et al*. (1994). To optimize the LM-PCR reactions, the DNA was purified by a combination of phenol-chloroform extraction and ethanol precipitation, after both the first-strand synthesis and the ligation reaction.

Cytoplasmic mRNA isolation and Northern blot Total cytoplasmic RNA was first isolated following the RNeasy Mini Protocol (Qiagen). Then, cytoplasmic mRNA was isolated from this total cytoplasmic RNA using Oligotex suspension (Qiagen). 2–3 μg of cytoplasmic mRNA was run on a 0.8% formaldehyde-agarose gel and blotted to a Zeta-Probe nylon membrane (Bio-Rad). The blot was probed with the random primer-^{32}P-labeled *Bsp*HI-*Nde*I DNA fragment (~440 bp, near the 5′-*Xho*I site). The blot was stripped and reprobed with a human G3PDH cDNA (Clontech) as a control.

Results and Discussion

Comparison of DNase I hypersensitive sites between pre-B and plasma cells The DNase I hypersensitive sites (HSSs) 1–6 in the upstream of J chain gene were shown to correlate with its gene expression when the HSSs were assessed from immature B to plasma cells representing each stage of B cell development (Kang *et al*., 1998). Surprisingly, when we extended DNase I hypersensitivity assay further into pre-B cell lines such as PD36 to correlate the HSSs with J chain gene expression, we found that the HSSs 3–4 were open despite of the absence of J chain gene expression in this cell line (Fig. 1A, top blot). However, consistent with the absence of J chain gene expression in PD36 cells, the HSS1 containing its promoter was shown to remain in a closed state (Fig. 1A, bottom blot). The same results were obtained when we tested another pre-B cell line, PD31 (data not shown). The open HSSs 3–4 in these pre-B cell lines might not be due to a chromosomal translocation and/or viral integration, because the genomic DNA structure in the vicinity of J chain gene was shown stable in all the cell lines tested by genomic restriction mapping analysis (data

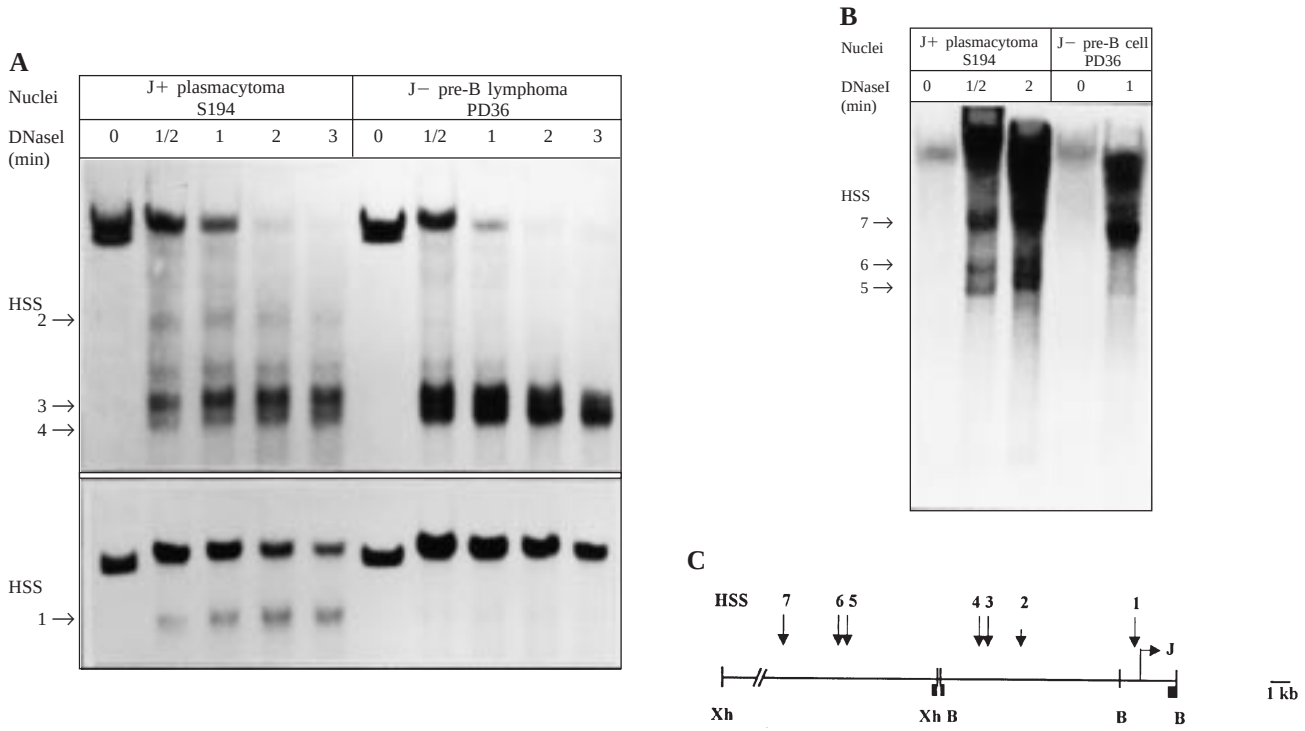

Fig. 1. Differential opening of chromatin in the upstream of J chain gene at the pre-B and plasma cell stages. **A.** Southern blot analysis of HSSs 1–4 in PD36 and S194 cells. Nuclei isolated from the cells were digested with DNase I for the times indicated. The DNA was extracted, digested with *Bam*HI, and assayed by Southern blots. HSSs 2–4 were probed with the 165 bp *Bam*HI-*Dra*I sequence from the 5′ terminus of the 8.2 kb *Bam*HI fragment. The same stripped blot was reprobed for HSS1 with the 600 bp *Eco*RI-*Bam*HI sequence from the 3′ end of the 2.8 kb *Bam*HI fragment. **B.** Southern blot analysis of HSSs 5–7 in PD36 and S194 cells. Southern blot analysis was performed by the procedure as described in (**A**) except *Xho*I was used instead of *Bam*HI. The probe was 310 bp sequence from the 3′ end of the 23 kb *Xho*I fragment. **C.** Map showing the locations of restriction sites, probes, and HSSs in the upstream of J chain gene. The thick bars at the ends of restriction fragments indicate the respective probes. B, *Bam*HI; Xh, *Xho*I.

not shown). Ultimately, however, the confirmation of the open HSSs 3–4 in normal pre-B cells might be necessary, although normal pre-B cells would be quite challenging to obtain in amounts sufficient for this assay. Alternatively, pre-B cells could be obtained in sufficient amounts from the RAG1 deficient/heavy chain transgenic mice (Shaffer *et al*., 1997), although those pre-B cells might not yet be perfectly normal.

The open HSSs 3–4 are then closed in the next immature and mature B cell stages, and open again with the J chain promoter-containing HSS1 in the plasma cell stage for the J chain gene expression (Kang *et al*., 1998). Both the enhancer HSSs 3–4 and the promoter HSS1 are induced open by the IL-2 signal, possibly via STAT5, in the presecretor BCL_1 cells for the expression of J chain gene and then maintained open constitutively in the plasma cells (Blackman *et al*., 1986; Kang *et al*., 1998; Minie and Koshland, 1986). However, in pre-B cells, HSSs 3–4 were open while the HSS1 remained in a closed state. Furthermore, we noticed the absence of HSSs 5–6 and the presence of two HSSs of slightly different mobility around HSS7 (major faster one and minor slower one) in PD36 pre-B cells as compared to those of S194 plasma cells (Fig. 1B). However, the major faster one (around 9 kb) in PD36 might correspond to HSS7 because we experienced a little irregular migration of the long DNA in agarose gel, depending on the sample preparation, and HSS7 was shown to be constitutively open in all the other cells tested. In any case, the pattern of HSSs in pre-B cells might be caused by an early B cell developmental signal other than IL-2, because PD31 and PD36 represent the IL-2-irrespective pre-B cell stage. The pattern of HSSs in pre-B cells prompted us to search for a pre-B cell-specific expression of a gene in the vicinity of J chain gene as a potential target of the open enhancer HSSs 3–4 (see below).

Comparison of DNase I hypersensitivity in the vicinity of J chain gene between B and non-B cells showed that the DNA in non-B cells such as EL4 T cells or Ltk^- fibroblast cells was completely inaccessible by DNase I, indicating a highly condensed state (data not shown). However, the chromatin in the vicinity of J chain gene in B cells appeared to exist as a slightly decondensed form, as compared to that in non-B cells. This slightly decondensed

chromatin in B cells might represent a poised chromosomal state ready for dynamic opening and/or closing of the HSSs during B cell development, and for the quick responses to the opening signals for the expression of two stage-specific genes. The dynamic regulation of chromatin structure in the vicinity of J chain gene during B cell development could provide an excellent model system for the study of B cell differentiation and its gene expression in the context of chromatin structure.

Dimethyl sulfate (DMS) *in vivo* footprinting in the pre-B cells The open enhancer HSSs 3–4 in the pre-B cells were further analyzed by performing DMS *in vivo* footprinting within the core 0.5 kb region to see if there might be any differential binding of trans-acting factors in these cells as compared to that of the plasma cells. The comparison showed a pre-B cell-specific footprint in addition to the common ones seen at two E2 boxes (E2A sites) and two CBF (core binding factor) sites (Clevers and Grosschedl, 1996) (Fig. 2). The pre-B cell-specific footprint turned out to be over a potential EBF (early B cell factor) site (11 out of 14 match to the consensus) (Travis *et al*., 1993). EBF and E2A have been reported as the important regulatory transcription factors in the early B cell development (Bain *et al*., 1997; Lin and Grosschedl, 1995). Furthermore, exogenous EBF and E47 were reported to collaborate to induce expression of the endogenous genes from the silent *λ5* and *VpreB* loci (Sigvardsson *et al*., 1997). With regard to these points, our *in vivo* footprints seen at the potential EBF and E2A sites within HSSs 3–4 could be related to both a chromatin-opening signal and an enhancer activity for the pre-B cell-specific expression of a gene (see below) in this early B cell stage. We also tried DNase I *in vivo* footprinting to see other footprints that might be missed by DMS method. However, the DNase I method was not as good as the DMS method.

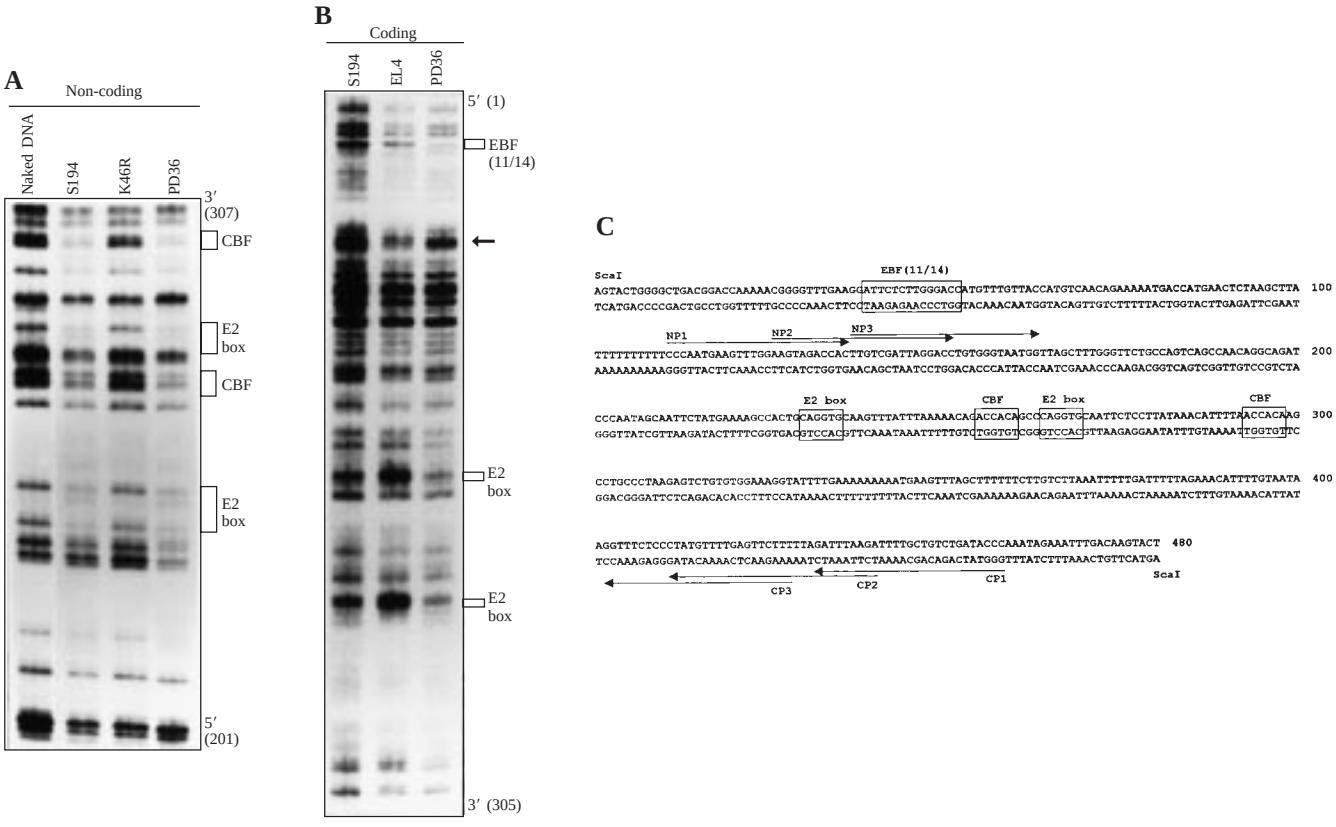

Fig. 2. Differential factor binding within the core enhancer HSSs 3–4 at the pre-B and plasma cell stages. (**A** and **B**) DMS *in vivo* footprinting of the core enhancer sequence shown in (**C**). EL4 and K46R as the chromosomal DNA controls, which showed almost the same pattern of DMS-bands as the naked DNA control, are a mouse T cell line and a mouse mature B cell line, respectively. PD36 is a mouse pre-B cell line, and S194 is a mouse plasmacytoma cell line. The naked DNA as another control is from S194 cells. The footprints are bracketed on the right sides of the autoradiographs. Other minor footprints seen around the E2 boxes were not consistently observed. The arrow on the right of panel B indicates a DMS-hypersensitive band at nucleotide number 128. **C.** The sequence of 0.5 kb *Sca*I core enhancer DNA fragment (GenBank accession number: AF122014). The footprinted consensus sequences are boxed. Primers for *in vivo* footprinting are indicated by arrows (NPs 1–3 for the noncoding strand and CPs 1–3 for the coding strand). Nucleotide position is numbered from the 5′ *Sca*I site.

Pre-B cell-specific expression of a gene in the upstream of HSSs 3–4 Because PD31 and PD36 pre-B cells had the open enhancer HSSs 3–4 despite the closed J chain gene promoter HSS1, we tested a possibility that its neighboring genes might be regulated by this open enhancer in the pre-B cell stage. When we searched for any potential neighboring gene expression by performing northern blot hybridization using nearby genomic DNA fragments as probes, a PD36-specific transcript was detected in the blot with a probe near the 5′ *Xho*I site, which is located about 23 kb upstream of HSSs 3–4 (Fig. 3). This transcript was also detected in PD31 cells (data not shown). However, the transcript was not detected in K46R mature B and S194 plasma cell lines. We speculate that the pre-B cell-specific gene for this transcript might be regulated by the open enhancer HSSs 3–4 in the pre-B cell stage. The speculated correlationship between HSSs 3–4 and pre-B cell-specific gene expression could be verified by the future cloning of this pre-B cell-specific gene.

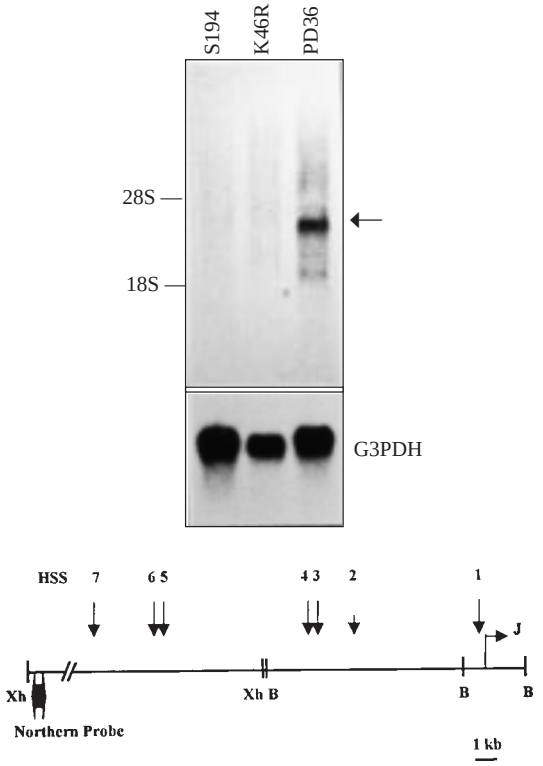

Fig. 3. Northern blot for the identification of a pre-B cell-specific gene expression. Cytoplasmic mRNA was size-fractionated in a 0.8% formaldehyde/agarose gel. The blot was hybridized with the 440 bp *Bsp*HI-*Nde*I fragment near the 5′ end of the 23 kb *Xho*I DNA fragment, the location of which is indicated with a thick two-headed arrow in the map below the blot. The arrow on the right side of blot indicates a transcript that is detected only in PD36 cells. The positions of 28S and 18S ribosomal RNA are indicated on the left side. The same stripped blot was reprobed with a control hG3PDH cDNA.

Table 1. Summary of chromatin structure, and expression of a pre-B cell-specific gene and J chain gene during B cell differentiation.

	Stages in B cell differentiation			
	Pre-B	Immature B	Mature B	Plasma cell
Lymphoid cell line	PD36	WEHI231	K46R	S194
Chromatin structure[a]				
HSS1	−	−	−	+
HSSs 3–4	+	−	−	+
HSSs 5–6	−	−	−	+
HSS7	+	+	+	+
Pre-B cell-specific gene	+	nd	−	−
J chain gene[b]	−	−	−	+

nd, not determined.
[a] Data for WEHI231 and K46R are from Kang *et al.* (1998).
[b] Data from Koshland (1985) and Rinkenberger *et al.* (1996).

Expression of the pre-B cell-specific gene, J chain gene expression and chromatin structure on the HSSs during B cell development are summarized in Table 1. Furthermore, because two neighboring genes might seem to be regulated differentially during B cell development by the common enhancer-containing HSSs 3–4, the chromosomal regulatory elements such as boundary element/insulator could be suggested between the two genes (Corces, 1995).

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