Bone Marrow Cells in X-linked Agammaglobulinemia Express Pre-B-specific Genes (λ-like and V Pre-B) and Present Immunoglobulin V-D-J Gene Usage Strongly Biased to a Fetal-like Repertoire

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Abstract

Expression of Ig and Ig-related genes has been studied in bone marrow cells from two patients with severe form of X-linked agammaglobulinemia (XLA). Phenotypic analysis revealed the presence of pre-B cells, in the absence of mature B cell markers. The pre-B-specific genes, λ-like and V pre-B, were normally transcribed. Sequence analysis of 48 distinct V-D-J cDNA clones directly derived from XLA bone marrow cells indicated that they had characteristics of an early fetal pre-B repertoire. All VH families were identified, with a strong bias in the gene usage: a few VH genes were largely overexpressed, either germline or slightly mutated; most genes had been located 3′ of the VH locus and were also used in fetal liver (8–13 wk of gestation). Short D regions, (resulting from D-D fusion, making usage of all D genes in both orientations with utilization of the three reading frames), restricted N diversity, and a fetal JH usage pattern were also observed. Taken together, our data suggest that the XLA defect does not alter V-D-J rearrangements nor the expression of μ, λ-like, and V pre-B transcripts and most likely results in a poor efficiency of some critical steps of the B cell maturation. (*J. Clin. Invest.* 1993. 91:1616–1629.)
Key words: immunoglobulin • immunoglobulin-related gene expression • primary immune deficiency

Introduction

X-linked agammaglobulinemia (XLA)[1] is a primary immune deficiency characterized by a severe impairment of immunoglobulin synthesis correlated with a low number of mIgM-positive B lymphocytes and by the lack of plasma cells (reviewed in references 1 and 2). Analysis of the X chromosome inactivation in XLA carrier women has shown that the defect was specific to the B cell lineage (3–7). The exact nature of the defect is, however, not known. B precursors characterized by early markers such as TdT, CD10, and CD19 (8) are present in the bone marrow. Although truncated heavy chains have been identified earlier (9), the recombinases that direct Ig and T cell receptor (TCR) gene rearrangement do not seem to be affected by the XLA gene since the T cell compartment appears to be normal (8). More recent reports have shown that the IGH locus could recombine, and that some Epstein-Barr virus (EBV)-transformed cell lines could be derived from XLA patients, suggesting that block of the B cell maturation was leaky (3, 10–13).

Ig gene expression necessitates rearrangements that occur according to an apparently strict ordered program H → κ → λ (14). Studies using transgenic mice (15) and gene targeting (16) have clearly shown that each step is controlled by the successive Ig gene products in their membrane form. Expression of the μ chain at the surface of pre-B cells has been shown to take place through the so-called μ–ψL complex in which the regular L chain is replaced by the product of two genes, specifically expressed in pre-B cells, termed V pre-B and λ-like (17–22). Because the XLA defect seems largely characterized by an impairment of the early steps of B cell differentiation, we have studied the expression of Ig and Ig-related genes in non transformed bone marrow cells isolated from XLA patients. Our results indicate that there is no major impairment at the pre-B stage. The λ-like and V pre-B genes are expressed, and the H chain repertoire makes use of all VH families, with a pattern of expression that resembles that of the fetal tissues. This observation, taken together with the fact that some level of light chain rearrangement takes place, suggests that the XLA defect most likely resides in a poor efficiency of a critical step of the B cell maturation, independent of the Ig gene recombination machinery.

Methods

Cases report. Bone marrow from two XLA patients was used in this study. Patient L.E. was a 3-yr-old boy with recurrent otitis and gastroenteritis from the age of 3 mo. Blood cell counts showed neutropenia and normal amount of lymphocytes. Serum immunoglobulin levels were below detection threshold (0.07 mg/ml for IgG, IgM, and IgA). No B cell, as appreciated from the lack of mIgM positive cells, was found. Diagnosis of XLA was confirmed by the X inactivation pattern analysis of the mother cells using methylation sensitive enzyme and restriction fragment length polymorphism (RFLP) study (5). A skewed pattern of X inactivation was observed in the mother EBV-derived B cell lines while T lymphocytes and polymorphonuclear cells displayed the expected random pattern. Patient R.S. was a 20-yr-old man with recurrent upper and lower respiratory tract infection. Serum immunoglobulin levels were below detection threshold and no mIgM-positive cells were detected in blood samples.

Surface marker analysis. Cells were isolated from freshly drawn heparinized bone marrow by means of Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. Flu-

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Received for publication July 8, 1992 and in revised form 29 October 1992.

1. *Abbreviation used in this paper:* XLA, X-linked agamma-globulinemia.

J. Clin. Invest.

0021-9738/93/04/1616/14 $2.00
Volume 91, April 1993, 1616–1629

orescence staining was performed using the following antibodies: CD19 (IOB4, IgG1), CD21 (IOB1a, IgG1), CD22 (IOB22, IgG1), CD24 (IOB3, IgG1), CD34 (IOM34, IgG1), from Immunotech, Marseille, France and anti-mIgM (Caltag, San Francisco, CA). Direct fluorescence staining was performed using PE- or FITC-conjugated mAb (CD19, CD34). Indirect immunofluorescence staining (CD21, CD22, CD24) was performed using a FITC- or PE-conjugated goat anti-mouse Ig (Gamig, Nordic, Tilburg, The Netherlands). The analysis was performed using a FACSCAN (Becton, Dickinson & Co., Mountain View, CA), on cells gated to exclude myeloid cells.

Preparation of cDNAs. Extraction of RNA from 2×10^6 bone marrow XLA or normal cells was performed by the guanidinium thiocyanate method followed by centrifugation in cesium chloride solution, according to Sambrooke et al. (23), as indicated in detail elsewhere (24). cDNA was prepared from 2 μg of total RNA, using 0.8 μg of oligo dT as primer and the reverse transcriptase of Bethesda Research Laboratories (Cergy-Pontoise, France) used according to the manufacturer's instructions.

PCR analysis. PCR was performed in a Perkin-Elmer Cetus (Norwalk, CT) apparatus, following the manufacturer's instructions, and using 500 ng of cDNA with 50 pmol of the appropriate oligonucleotides. Amplified products were run in agarose gel made 2% in 1× 0.089 M Tris, 0.089 M Borate, and 2.5 mM EDTA (TBE) and transferred on Hybond N membrane in 20× SSC and characterized upon hybridization with specific probes.

Experimental conditions used for the analysis of the pre-B–specific gene expression were previously described using non saturating substrate concentrations, i.e., between 100 and 500 ng of RNA (24) and led to the identification of specific fragments of 210 and 190 bp for V Pre-B and λ-like amplification products, respectively. Normalization by reference to normal bone marrow cells was introduced using amplification of actin RNA. In addition, for each series of PCR, control samples of RNA from one positive cell (Nalm-6, a pre-B cell line) and two negative cells (1SP3, an EBV-derived cell clone and Jurkat, a T-cell line) were run in parallel. A blank containing no input RNA was also included.

Analysis of the κ transcripts was performed following the same conditions, using in separate experiments oligonucleotides corresponding to the four different family-specific Vκ segments (25) and to the 5′ terminal section of the Cκ coding sequence (linker sequences are italicized):

Vκ1: (5′*ATCCGCGG*ACATCCAGATGACCCAGTC3′)

Vκ2: (5′*ATCCGCGG*ATATTGTGATGACTCAGTC3′)

Vκ3: (5′*ATCCGCGG*AAATTGTGTTGACGCAGTC3′)

Vκ4: (5′*ATCCGCGG*ACATCGTGATGACCCAGTC3′)

Cκ: (5′*ATGCGGCCGC*GGGAAGATGAAGACAGATG3′)

The amplified products were checked with a Jκ probe. A blank containing no input RNA was also included.

Construction of cDNA libraries and identification of the cDNA clones. VH transcripts from patient R.S. were cloned after anchored PCR. cDNA between 0.5 and 2 kb were purified by electrophoresis on 0.8% low melting point agarose in 1 × TBE and oligo dG tailing was performed as described by Loh et al. (26) with TdT (Bethesda Research Laboratories) for 20 min at 37°C. Amplification was performed with 2.5 units of Taq polymerase (PROMEGA) in 100 μl of the standard buffer and 50 pmol of each oligomer (linkers are italicized):

linker-oligo dC:

(5′*ATGCATGCCGCGGCCGC*CCCCCCCCCCCCC3′)

Cμ linker:

(5′*ATCCGCGGCCGCGG*AATTCTCACAGGAGACGA3′)

for 30 cycles in a Perkin Elmer Cetus apparatus with the following program: first cycle 94°C, 3 min; 50°C, 4 min; 72°C, 2 min; 10 cycles 94°C, 1.5 min; 50°C, 4 min; 72°C, 2 min; 19 cycles 94°C, 1.5 min; 55°C, 4 min; 72°C, 2 min except for the last cycle for which the final extension at 72°C proceeded for 10 min. The amplified material was separated on 1% low melting point agarose gel and the fraction contained within the 0.3- and 0.6-kb range was isolated. One-tenth of this material was amplified with the same primers for 40 cycles at 58°C. Amplified products were precipitated and the resulting DNA was digested with NotI, ligated in NotI-treated and dephosphorylated bluescript vector (Stratagene, Inc., La Jolla, CA). Transformation was performed in JM 101 by electroporation (27). Resulting colonies were screened with the JH probe.

VH transcripts from patient L.E. were cloned by PCR using, in separate experiments, oligonucleotides corresponding to the different family specific VH leader segments and to the 5′ terminal section of the Cμ coding sequence (linkers are italicized):

VH1:

(5′*AAAGCGGCCGC*ATGGACTGGACCTGGAGGGTC3′)

VH2/VH4:

(5′*ATCCGCGGCCGC*ATGAAACACCTGTGGTTCTT3′)

VH3:

(5′*AAAGCGGCCGC*ATGGAGTTTGGGCTGAGCTGG3′)

VH5:

(5′*AAAGCGGCCGC*ATGGGGTCAACCGCCATCCTC3′)

VH6:

(5′*AAAGCGGCCGC*ATGTCTGTCTCCTTCCTCATC3′)

Cμ:

(5′*ATCCGCGGCCGCGG*AATTCTCACAGGAGACGA3′).

for 30 cycles in a Perkin-Elmer Cetus apparatus with the following programme: first cycle: 94°C, 3 min; 60°, 3 min; 72°, 3 min; 29 cycles: 94°, 1.5 min; 60°, 3 min; 72°, 3 min except for the last cycle for which the final extension at 72°C proceeded for 10 min. The amplified material was separated on 1% low melting point agarose gel and the fraction at 0.45 kb was isolated. One-tenth of this material was amplified with the same primers for 30 cycles at 60°C. Amplified products were digested with NotI, and cloned as above. Resulting colonies were screened with the VH-specific probes.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination protocol (28) using ^{35}SdATP and a DNA sequencing system-T7 DNA polymerase (Amersham International, Amersham, UK). Inserts were sequenced using T3 and T7 oligonucleotides.

DNA probes. IgCλ is a genomic 0.7-kb EcoRI-HindIII fragment containing the Cλ3 gene (29) obtained from Dr. M.-P. Lefranc (Montpellier II University, Montpellier, France) and was used to identify the cross-reactive λ-like gene product. V pre-B is an amplified product from amino acid residues 50–120 of the coding region (22). JH (0.5 kb SacI) (30) and Jκ (2 kb SstI) (31) genomic probes were gifts from Dr. P. Leder (Harvard Medical School, Boston, MA). VH1 (0.3 kb PstI-EcoRI from pVE3-D10) and VH2 (1.2 kb EcoRI-EcoRV from pVCE-1) genomic probes were obtained from Dr. T. Honjo, Faculty of Medicine, Kyoto, Japan (32). VH3 (0.32 kb EcoRI-HaeIII from γ F9 cDNA (33), VH4 (0.33 kb PstI from 1-911), VH5 (0.38 kb HincII-PstI from 5-1R1), and VH6 (0.3 kb EcoRI-StuI from 6-1R1) genomic probes were given by Drs. J. Berman and F. Alt, Howard Hughes Medical Institute, Boston, MA (34).

Results

Phenotypic characterization of bone marrow XLA cells. FACS analysis of bone marrow cells from one patient (L.E.) is given

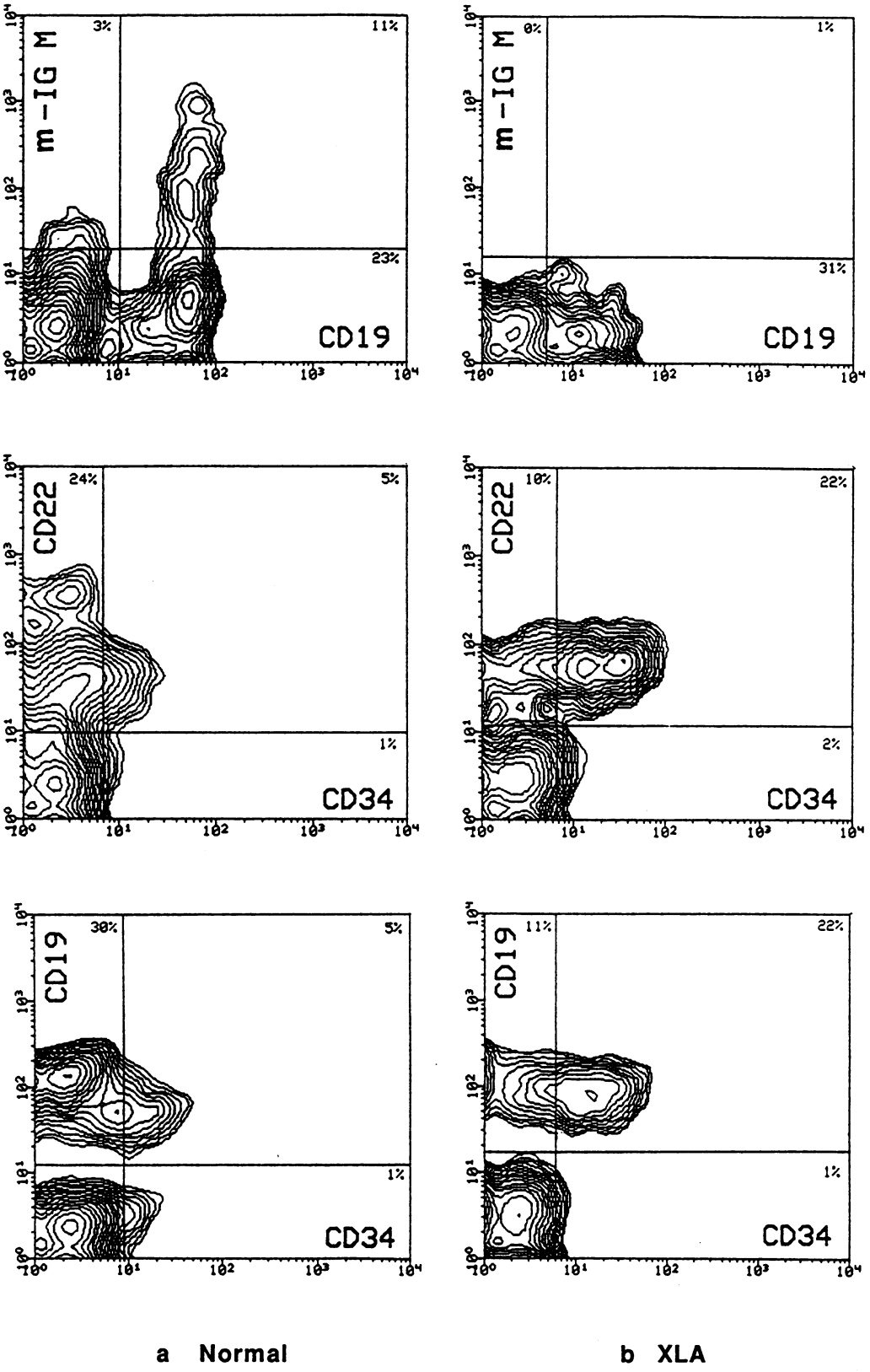

Figure 1. FACS analysis of (*a*) normal and (*b*) XLA bone marrow cells. CD19, CD34, mIgM were identified respectively with PE-conjugated IOB4, FITC-conjugated IOM34, and anti-IgM by direct immunofluorescence. CD22 was tested with IOB22 by indirect immunofluorescence using a FITC-conjugated GAMIG. Fluorescence intensity was expressed on a log scale. Analysis was performed on cell gate to exclude myeloid cells.

in Fig. 1. No mIgM was detected, whereas ~ 33% of the cells was found positive for CD19 and/or CD22. From these, 66% were found double-positive with respect to the CD34 marker, as opposed to 14% in normal bone marrow cells. By contrast all XLA cells were negative for CD21 and CD24 (33% and 37% in the controls, respectively, data not shown). In addition, immunofluorescence revealed some cells with intracytoplasmic μ chains (9/500 mononuclear cells) whereas no intracytoplas-

mic light chain ($\kappa + \lambda$) was detected. Cells from patient R.S. were checked only for mIgM, and were all found negative.

Expression of the λ-like and V pre-B genes. To normalize the amount of RNA used for λ-like and V pre-B amplification from both XLA and normal bone marrow cells, we first performed a PCR analysis using serial dilutions of RNA and actin-specific oligonucleotides (data not shown). Ranges covering 500–100 ng from both RNAs were then amplified with λ-like or V pre-B specific oligonucleotides. In Fig. 2 *a* is reported the λ-like PCR pattern obtained for 500, 400, and 300 ng of both cDNAs. Specific bands were identified at 190 bp with the same decrease in intensity for the two samples. No signal was recovered at lower concentrations, as already reported (24). V pre-B and λ-like PCR patterns obtained with 500 ng of normal and XLA bone marrow cells are given in Fig. 2 *b*. The 210-bp (VpreB) and 190-bp (λ-like) specific bands have similar intensities, whichever their origin. Similar results were obtained for the two XLA patients clearly suggesting that the two pre-B specific genes were normally expressed on a quantitative basis in XLA patients.

Analysis of the V-D-J repertoire in XLA bone marrow cells. RNA from Ficoll purified bone marrow cells from patient R.S. was amplified by anchored PCR using a 5′Cμ-specific oligonucleotide, yielding nine different sequences, of which six were of the V-D-J type with variable VH lengths, one was of the D-J-Cμ form (RS 54), and two (RS 39 and RS 51) for which presence of VH residues could not be determined due to the limited size of the transcripts (Table I). In order to select for complete transcripts, RNA from patient L.E. was amplified using 5′Cμ and each of the leader VH-specific oligonucleotides. 143 clones were sequenced, from which 39 different V-D-J sequences were identified and are listed according to the VH family grouping in Table I. Most sequences were obtained several times from different clones (between two and nine), with identical results. The complete sequences have been deposited at the EMBL data bank (accession nos. X65883–X65920).

It can be observed that all VH families are used except VH2, which was earlier shown to be poorly expressed in the normal Ig repertoire (38). Within each family, all transcripts were assigned to one of the known germline genes, taken from EMBL data bank or from recently published data (36) as indicated in Table I. The number of nucleotide substitutions necessary to derive the cDNA sequences from the known germline genes is also indicated. In most cases, fewer than 10 mutations, that occurred randomly all along the VH segment, were sufficient to derive most expressed sequences from the germline genes. 40% of the transcripts had two mutations or fewer, including six sequences that were completely germline. Alternatively, minor nucleotide substitutions may be due to allelic polymorphism as illustrated by comparison with the data of Shin et al. (35). On the basis so defined, gene usage within each family clearly appears restricted. For instance, in the VH1 family, six different germline genes accounted for the 13 reported sequences, of which gene 1-3b (35) was encountered six times. Four genes were used in a total of 11 VH4 sequences, V 79 being present seven times (37).

Sequences encompassing the CDR3 regions are presented in Fig. 3 according to the JH usage. Assignment of D segments was defined using homology with direct and reverse D (40–42) and DIR (DH gene containing irregular spacer signals [43]) germline sequences on the basis of a minimal length of six identical nucleotides or seven nucleotides with no more than one mismatch. It can be seen on Fig. 4 that the expressed D segments could be derived from one single germline gene (47% of the sequences) to up to four D genes resulting from multiple fusions. Both direct and reverse sequences were identified, with occasional usage of DIR genes. Three sequences did not match any of the known D genes. Most D genes were expressed (Table II), of which 27% in the reverse orientation. The DXP family accounted for 38% of the total. The three reading frames were used, although reading frame 2 was used more frequently.

Detailed organization of the CDR3 regions is given in Fig. 3. N sequences were introduced whenever no nucleotide counterpart could be identified on either of the V, D, or JH germline segments. Finally, whenever nucleotides could undifferently fit with 3′V or 5′D and/or with 3′D or 5′J, they were arbitrarily assigned to V or to J, respectively. On these grounds, N diversity, with possible P diversity (39), appeared very limited, since it was absent in over 40% of either the V-D and the D-J junctions. When present, the N diversity segment averaged four

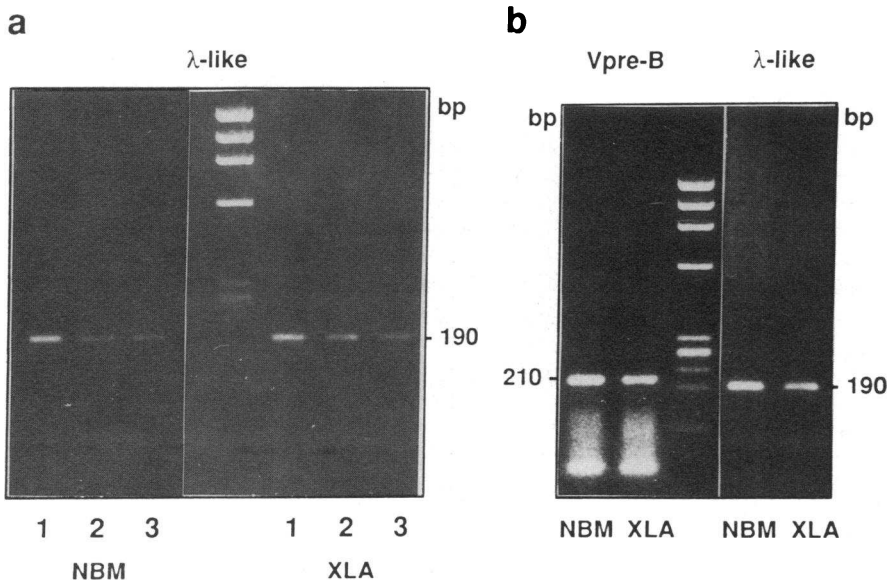

Figure 2. Expression of the λ-like and V pre-B genes in XLA and normal bone marrow cells (*NBM*), by PCR analysis. (*a*) λ-like amplification using 500 (*1*), 400 (*2*), and 300 ng (*3*) of RNA. (*b*) V pre-B and λ-like amplification using 500 ng of RNA. PCR products were detected after electrophoresis on 2% agarose gel by ethidium bromide staining. Sizes were estimated from the ΦX174 -Hae III fragments.

Table I. Main Characteristics of XLA μ Transcripts

	VH	N	DH	N	JH	Length N-D-N	Length CDR3	I/O
LE 1-6	1* 1-4b‡ (3)§				6	0	30¶	in**
RS 1-103	1 1-8 (1)		D23/7		3	7	27	out
LE 1-17	1 1-3b (1)		DIR‡‡		3	8	30	in
LE 1-25	1 1-46 (5)		DHFL16	5	3	13	30	in
LE 1-54	1 1-46 (5)		DHFL16	5	3	13	30	in
LE 1-10	1 1-3b (5)		DHFL16	5	3	13	30	in
LE 1-100	1 1-46 (6)		DHFL16	5	4	13	30	in
LE 1-9	1 1-3b (0)		DHFL16	6	4	14	27	in
RS 1-92	1 partial	8‖	DHQ52	2	3	17	36	in
LE 1-82	1 1-3b (2)	2	DK4‡‡-DM2‡‡		6	18	42	in
LE 1-29	1 1-2 (7)	3	DIR1-DLR4‡‡		4	20	33	in
LE 1-42	1 1-3b (0)	6	DK1		6	20	51	in
LE 1-76	1 1-3b (0)		DIR1-D23/7-DK1-DXP'1		4	31	51	in
LE 1-23	1 1-3 (4)		deleted		deleted			
LE 3-1	3 3-13 (4)		unknown		6	4	21	in
LE 3-2	3 3-30 (2)		unknown		6	4	21	in
LE 3-13	3 3-7 (3)		DK1	1	6	8	33	in
RS 3-128	3 partial	2	DK4-D21/7		4	19	39	
LE 3-21	3 3-30 (2)	2	D21/7‡‡-DHQ52	6	4	24	39	out
LE 3-12	3 3-23 (4)		D21/7-DIR1‡‡		4	25	42	in
LE 3-11	3 partial	6	DN1-D21/05	3	4	25	42	
LE 3-20	3 3-23 (7)	6	DN1-D21/05	3	4	25	42	in
LE 3-16	3 3-64 (9)	4	DXP4	1	4	26	42	in
LE 3-15	3 3-11 (9)	11	D21/9	5	3	35	54	out
LE 4-5	4 V79 (7)	1	DHFL16		6	12	42	in
LE 4-30	4 V79 (2)	3	DHFL19		5	12	36	in
LE 4-89	4 4-39 (8)		unknown		3	20	42	in
LE 4-103	4 V11 (7)	3	D21/9		3	21		
LE 4-63	4 V79 (10)	3	DIR1‡‡	1	4	22	38	in
LE 4-104	4 V79 (6)	3	D21/9	4	3	24	45	in
LE 4-8	4 V79 (4)		D21/7-D23/7	1	4	24	45	in
LE 4-34	4 V79 (10)	3	D21/9	4	3	24	45	in
LE 4-56	4 V79 (2)	2	D21/05	5	5	30	54	in
LE 4-105	4 4-39 (5)	9	DN4-DXP'1	7	3	38	66	in
LE 4-19	4 4-34 (1)	3	DLR2	9	6	42	66	in
LE 5-3	5 VH32 (4)	1	DLR5‡‡-DLR2	1	4	20	33	in
LE 5-11	5 VH32 (1)	2	DXP4-DHFL16		6	25	57	in
LE 5-7	5 VH32 (1)	4	‡‡DK4‡‡-DK4	1	4	28	46	out
LE 5-2	5 5-51 (0)	5	D21/10-DM1	7	3	31	51	in
LE 6-14	6 6-1 (1)	4	DM1‡‡		4	10	27	in
RS 6-91	6 6-1 (0)	4	DN1	6	6	21	48	in
RS 6-21	6 6-1 (2)	1	D21/9-D21/7	1	4	23	39	in
LE 6-16	6 6-1 (2)		DLR2‡‡-DN1-D22/12		6	28	57	in
LE 6-1	6 6-1 (0)	3	D21/7	5	6	36	69	in
RS 39	§§partial	2	DM1-DLR3‡‡		6	17		
RS 54	D-J		DHQ52-DLR3		4	22		
RS 51	§§partial		D21/9-DM2		4	32		
RS 12	§§partial	12	DLR1-DK1	10	6	40		

* Sequences are grouped according to the VH families.

‡ All germline gene sequences are taken from references 35 and 36 except V11 and V79 taken from reference 37 and VH32 taken from reference 38.

§ Number of mutations from the corresponding germline genes.

‖ Number of nucleotides in N region.

¶ Length of CDR3 was taken from residue 93 of the VH to the position preceding the TGG codon of the JH.

** I, in frame (in); and O, out of frame (out).

‡‡ D germline gene used in reverse orientation.

§§ VH family could not be assigned due to partial sequences.

	VH	N	D	N	JH	
RS 1-103	TGTGCGGGA		TGGCGTG		ATGCTTTTGATATCTGG	**JH3**
LE 1-17	TGTGCGAGAGA		GGGGTTGG		ATGCTTTTGATATCTGG	
RS 1-92	TGTGCGAGAGA	**T**AGGTGG**G**	CTGGGGA	CG	ATGCTTTTGATATCTGG	
LE 4-89	TGTGCGAGAGA		GGGCAGTGTCAAACCCCATG		ATGCTTTTGATATCTGG	
LE 4-104	TGTGCGAGAA	CAC	ACTATGATAGTAGTGGT	CTTG	ATGCTTTTGATATCTGG	
LE 4-34	TGTGCGAGAA	CAC	ACTATGATAGTAGTGGT	CTTG	ATGCTTTTGATATCTGG	
LE 4-105	TGTGCGAGAGA	**TC**TAGAGGG	GGAGAACGTCCTACTATGGCTC	CAAGATG	ATGCCTTTGATATCTGG	
LE 5-2	TGTGCGAGA	CGTCC	TTTGGGGGACTGGAACTAC	**G**GGGAAG	ATGCTTTTGATATCTGG	
LE 3-15	TGTGCGAGAGG	AGTGGACCGAT	TTACTATGATAGTATTGGT	CTCCG	GCTTTTGATATCTGG	
LE 1-25	TGTGCGAGAGA	**T**GACTAC**G**	CCGCA		TTTGATATCTGG	
LE 1-54	TGTGCGAGAGA	**T**GACTAC**G**	CCGCA		CTTGATATCTGG	
LE 1-10	TGTGCGAGAGA	**T**GACTAC**G**	CCGCA		TTTGATATCTGG	
LE 4-103	TGTGCGAGAA	CAC	ACTATGATAGTAGTGGTT			
LE 1-76	TGTGCGAGA		ATACCCCGTATTAGCAGTGGCTGGTTGGGGG		ACTACTTTGACTACTGG	**JH4**
RS 54			CTAACTGGGGA<u>T</u>TTGTGATGGT		ACTACTTTGACTACTGG	
RS 51			GTATTACTATGATAGTAGTGGTACCCGAAATA		ACTACTTTGACTACTGG	
LE 4-8	TGTGCGAGAGA		TTCGGGATTTTTGGAGTGGTTAT	C	CTACTTTGACTACTGG	
RS 3-128	TGTGCGAAAGA	**TC**	ATATAGCGGGAGCTACT		TACTTTGACTACTGG	
LE 5-7	TGTGCGAGA	**TC**GG	ACCTATGGCCGATACAGCTATGG	**A**	TACTTTGACTACTGG	
LE 3-12	TGTGCGAAAC		GGGGACTTAT<u>A</u>GCAGTGGCTGGTTT		CTTTGACTACTGG	
LE 3-11						
LE 3-20	TGTGCGAAAC	**G**GGACG	TTGCAGCAGTGGCTGG	**CGG**	CTTTGACTACTGG	
LE 6-14	TGTGCAAGAGG	**CTGG**	CCAGTT		TTTGACTACTGG	
LE 1-100	TGTGCGAGAGA	**T**GACTAC**G**	CCGCA		TTTGACTACTGG	
LE 4-63	TGTGCGAGAAA	CTC	CCTGGGCTGCTGCTGGGC	**A**	TTGACTACTGG	
LE 3-21	TGTGCGAGAGA	C**G**	CTCCCCGAACTGGGGA	**T**TGTTG	TTGACTACTGG	
LE 3-16	TGTGTGAAAGA	**T**TAC	TACGATTTTTGGAGTGGTTAT	G	TTGACTACTGG	
RS 6-21	TGTGCAAGAGA	**A**	TACTATGATAGT<u>C</u>TCGGGGTG	G	TTGACTACTGG	
LE 5-3	TGTGCGAGA	C	AAGAAGTAC<u>C</u>AGCTGCCA	**A**	TGACTACTGG	
LE 1-9	TGTGCGAGAG	GTAAACCC	TATATC		GACTACTGG	
LE 1-29	TGTGCGAAAG	GTT	CTCAGTG	GCTGGTACTA	GACTACTGG	
LE 4-56	TGTGCGAGAA	CC	TACGATATTTTGACTGGTTATTA	CCA**GT**	ACAACTGGTTCGACCCCTGG	**JH5**
LE 4-30	TGTGCGAGAAA	GGC	TGGTACCCC		CAACTGGTTCGACCCCTGG	
LE 5-11	TGTGCGAGA	CA	GTATTACGATTTTTTCCAGCCGG		ACTACTACTACTACGGTATGGACGTCTGG	**JH6**
LE 6-1	TGTGCAAGAGA	**TC**G	TTACTATGTTCGGGGGAGTTATTATAAC	TCTC**G**	CTACTACTACTACGGTATGGACGTCTGG	
LE 4-5	TGTGCGAGAG	G	GTCGCCGGGGG		ACTACTACTACGGTATGGACGTCTGG	
LE 1-42	TGTGCGAGAGA	**TCTTTT**	ATGCAGTGGCTGCG		ACTACTACTACGGTATGGACGTTTGG	
RS 12	TGTGCAAGA	GATCCCCGGGTA	GGTGGTGTA<u>G</u>CAGTGGCT	GGGACCTCTG	ACTACCACTACGGTATGGACATCTGG	
LE 1-6	TGTGCGAGGGA				CTACTACTACGGTATGGACGTCTGG	
RS 6-91	TGTGCAAGA	**T**GGT	CAGCAGCTGGT CCGGGT		TACTACTACGGTATGGACGTCTGG	
LE 6-16	TGTGCAAGAGG		AGCTCGCACCGGGTATAGCATTGGGACT		TACTACTACGCTATGGACGTCTGG	
LE 4-19	TGTGCGAGA	**TC**G	GGATATTGTAGTGGTGGTAGCTGCTACTCC	**GG**TCCGTGG	TACTACGGTATGGACGTCTGG	
LE 3-13	TGTGCGGGCGG	**C**TACGGT	G		ACTACAGTATGGACGTCTGG	
LE 1-82	TGTGCGAGAGA	AG	TAGCAGTGTC	TGGTTC	CTACGGTATGGATGTCTGG	
RS 39		GG	GGTATAACTACCACA		TACGGTATGGACGTCTGG	
LE 3-1	TGTGCGAGAGA		AAAG		ATGGACGTCTGG	
LE 3-2	TGTGCGAGAGA		AAAG		ATGGACGTCTGG	

Figure 3. Sequences of XLA-derived transcripts encompassing the CDR3 regions. Data are ordered by decreasing sizes of the various JH family segments. Possible residues for P diversity (39) are bolded. Putative N diversity at the D-D junctions is underlined.

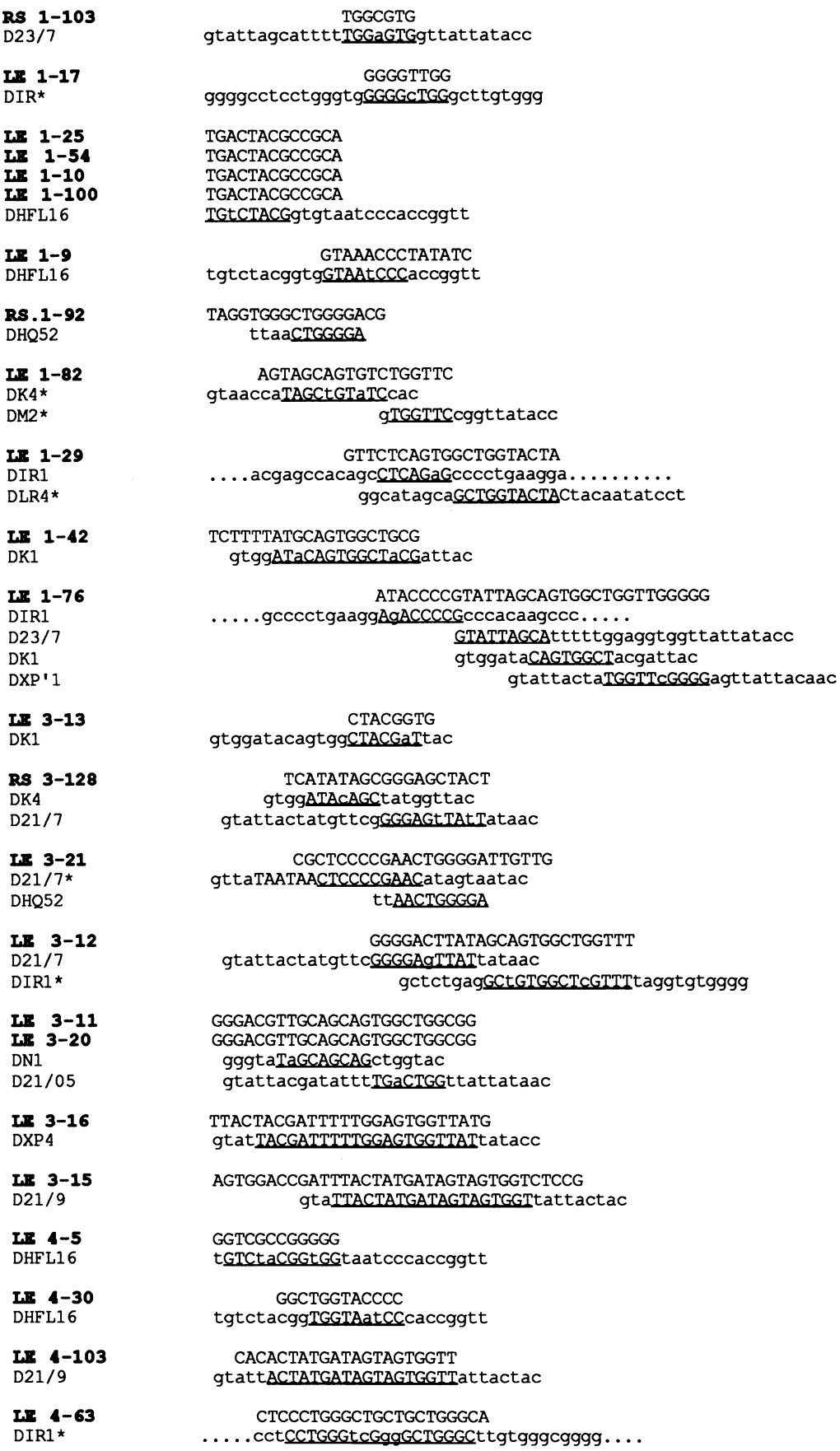

Figure 4. Identification of D germline genes from which transcripts originate. Portions of germline genes encountered in transcripts are underlined. Identities appear in upper cases, differences in lower cases. *Usage of germline gene in reverse orientation. Germline sequences are taken from references 40–43.

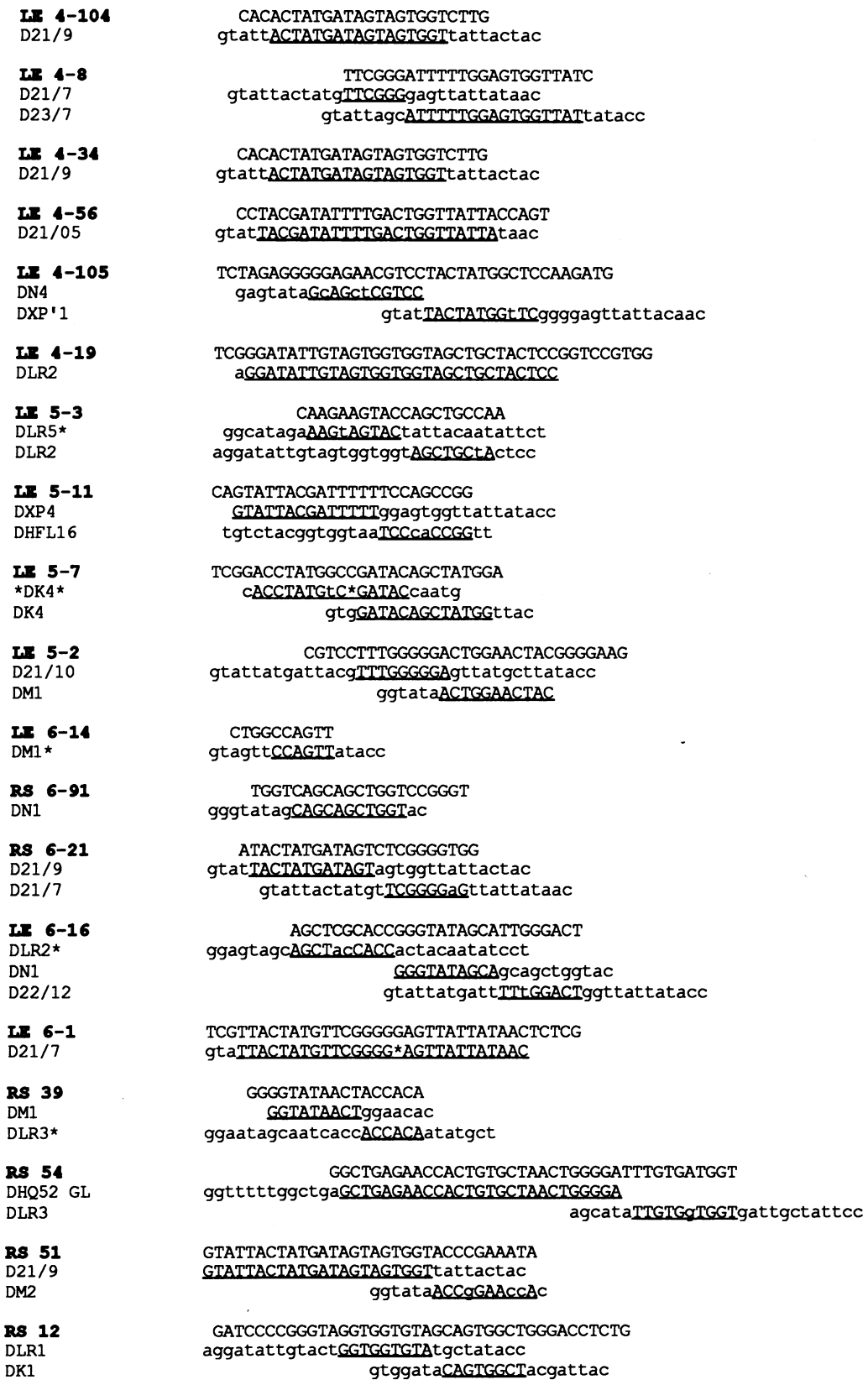

Figure 4. (Continued)

nucleotides and did not exceed 12. It cannot be excluded that the largest N regions might be encoded by so far undescribed D genes. In addition, possible N diversity (underlined residues in Fig. 3) could be defined at some D-D junctions, although very limited both in number (five cases) and size (one nucleotide). Length of the N-D-N segments extended from 0 to 42 nucleotides, with an average of 20–25 (Fig. 3 and Table I).

JH regions were found encoded by JH3 to JH6 genes, with

Table II. Frequency of Utilization of D, D Families, and D Reading Frame Genes in XLA μ Transcripts

	Direct*			Reverse‡				
	1st	2nd	3rd	1st	2nd	3rd	Gene usage§	Family usage‖
							%	%
1. DXP (32%)								38
D21/9		3					6	
D21/10	1					1	4	
D21/7	1	2	1	1			10	
D21/05		2					4	
D23/7	1		1				4	
D22/12		1					2	
DXP4		2					4	
DXP'1	2						4	
2. DLR (20%)								14
D5					1		2	
D4					1		2	
D3								
D2		1	1	1			6	
D1		1			1		4	
3. DA (12%)								2
DA1								
DA4				1			2	
4. DK (8%)								10
DK1	1		3				8	
DK4				1			2	
5. DN (8%)								8
DN1	1	2					6	
DN4			1				2	
6. DM (8%)								6
DM2					1		2	
DM1			1	1			4	
7. DFL16 (4%)		3	2				10	10
8. DHQ52 (4%)	1						2	2
9. DIR (8%)								10
DIR1 or DIR2		2		1	1	1	10	
Total	8	19	10	6	5	2		

Numbers in parentheses represent the expected frequency of utilization according to the size of each family. Individual genes within each family are listed from top to bottom according to their physical proximity to the JH locus. 1st, 2nd, and 3rd refer to the three reading frames. D gene references are from references 40–43.

* D gene in direct orientation.

‡ D gene in reverse orientation.

§ Frequency of gene usage of D segments in direct and reverse orientation.

‖ Frequency of D family usage.

various degrees of truncation of the 5′ ends (Fig. 3). Clone LE 4-103 was extensively truncated containing only the four last JH codons. Allelic variants used were JH3b, JH4b, JH5b, JH6b, and JH6c (44). Occasional mutations (one or two) were observed in 27% of the analyzed sequences.

CDR3 segments were defined from residue 93 of the VH (numbering of Kabat et al. [45]) to the position preceding the TGG codon of the JH genes. On this basis the CDR3 length varied from 21 to 69, with an average value of 41 (Table I).

Of the VDJ sequences 88% had an open reading frame (Table I). Although most V-D-J associations could be considered random, several peculiar cases were observed. For instance, the same V-D combination was found associated to different JH (LE 1-100 and LE 1-25). Conversely the same DJ was found associated to discrete VHs (LE 1-10 and LE 1-25; LE 4-34 and LE 4-104). Finally, two VDJ were using two discrete leader segments (LE 1-54 and LE 1-25). A few sequences were truncated, (LE 1-23 lacking DJ, LE 4-103 lacking most of the 5′ JH codons), or contained multiple deletions in VH (LE 3-11).

Expression of κ transcripts. κ transcripts could be identified in patient L.E.'s bone marrow cells after PCR using primers covering the four Vκ families and the 5′ end of Cκ. Bands of the expected size (between 320 and 340 bp, depending upon the Vκ families) were clearly detected, although of fainter intensity as compared to normal bone marrow cells (Fig. 5).

Discussion

The expression of Ig and pre-B specific-Ig-related genes was analyzed in bone marrow cells derived from two patients with a

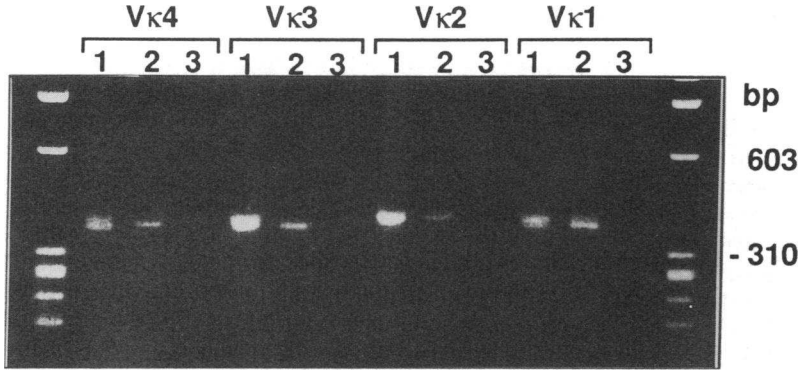

Figure 5. Expression of Vκ gene families in XLA and normal bone marrow cells, by PCR analysis, using 500 ng of RNA. PCR products were detected after electrophoresis on 2% agarose gel by ethidium bromide staining. Sizes were estimated from the ΦX174 -Hae III fragments. Lanes *1*, normal bone marrow; Lanes *2*, XLA bone marrow; Lanes *3*, no input RNA.

severe form of XLA. FACS analysis pointed to the pre-B nature of cells of the B lineage as appreciated from the absence of mIgM and of the CD21 and CD24 markers, characteristic of the B lymphocytes and the proportion of Cμ-positive cells that were found within normal limits. The presence of pre-B cells was confirmed through the expression of CD19 and/or CD22 in one third of the cells, of which 66% were also positive for CD34 (46). Double positive in normal bone marrow were only 14%, which is suggestive of a differentiation block somewhere along the pro-B → pre-B pathway.

Because some critical step necessary to the pre-B → B transition may be triggered by the μ-ψL complex (24, 47, 48), we first analyzed the expression of pre-B specific genes. Transcripts of the Ig-related genes, λ-like and V Pre-B, were identified by PCR, yielding fragments of the expected sizes (Fig. 2) and expressed in amounts that were similar to normal bone marrow. This indicates that pre-B cell differentiation can go along although the higher proportion of CD34-positive cells suggests a progressive decline in that process.

We next turned to μ chain expression. Anchored PCR, using an oligonucleotide primer specific for the 5′ end of the Cμ region revealed that most transcripts encompassed the V region (at least 66%), whereas 11% were clearly of the D-J-Cμ type. This already indicates that transcription is not limited to Dμ (9) or of the more recently described LS-Cμ transcripts (49). This finding prompted analysis of complete VDJ (Cμ) transcripts that were easily derived upon amplification with primers specific of the various VH-leader sequences and of the Cμ 5′ region. Detailed analysis of the 48 μ transcripts (Table I) revealed that all characteristics of an early "pre-B" functional repertoire were indeed present:

All VH families were identified (except VH2), including a marked contribution of VH5 and VH6, (50, 51). It can be observed that VH gene usage was biased in several ways: (*a*) 19 genes accounted for the 48 discrete V-D-J transcripts, indicating that some genes were used with a high frequency (Table I); (*b*) 16 out of these 19 genes were located in the 800 kb region covering the 3′ end of the VH locus, as schematically presented in Fig. 6, according to the map recently published by Matsuda et al. (36); (*c*) In Fig. 6, it can also be observed that most of the genes used in XLA were also expressed in a fetal liver as early as 8–13 wk of gestation (52). A somewhat similar gene usage in

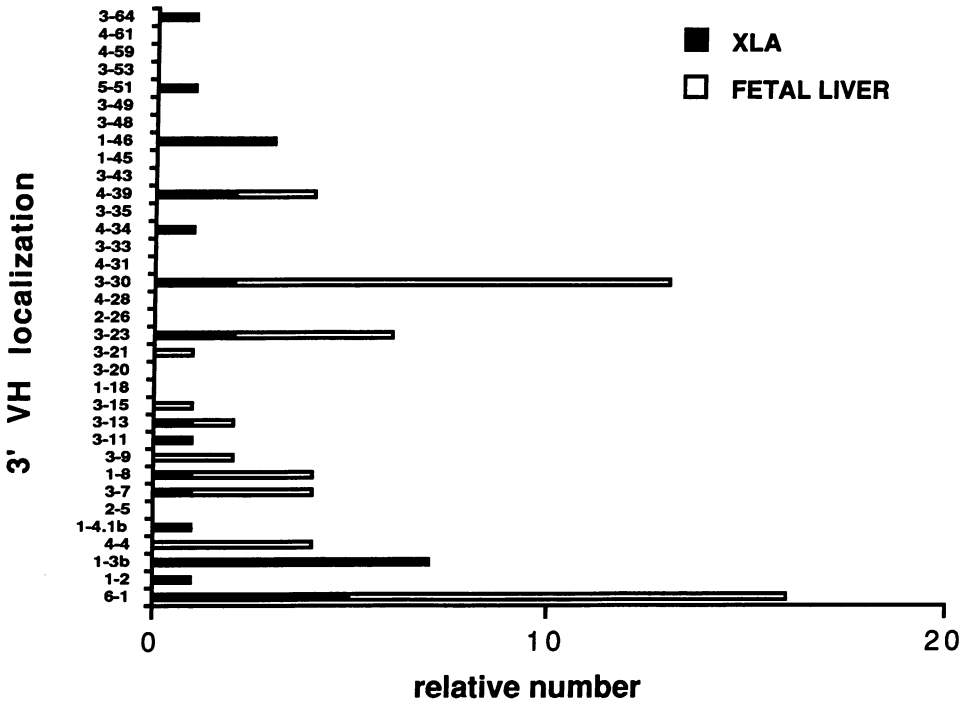

Figure 6. VH gene utilization in XLA bone marrow and fetal liver (52). Gene numbering (including functional genes only) on the *y*-axis is that of Matsuda et al. (36) and is presented from 5′ (top) to 3′ (bottom). Length of bars reflects the gene usage frequencies.

human fetal liver can be derived from data obtained by Schroeder et al. (53, 54) at 15 or 18 wk of gestation and is reminiscent of the preferential usage of the most 3′ VH gene early in mice ontogeny (55–57).

As outlined in Fig. 7, the average size of the XLA-derived D segments was close to those of the fetal liver between 8 and 13 wk of gestation (52) and significantly shorter than those of their adult PBL counterparts (44, 58). Same conclusions could be drawn from the comparisons of CDR3, indicating that D and CDR3 lengths were well correlated. They were also found shorter than those reported by Timmers et al. (12), and Anker et al. (11) who studied the XLA repertoire in EBV-derived clones which were found closer to that of adult PBL (Fig. 7). Besides their distinctive lengths, the D regions described by Timmers et al. (12) do not differ from the other characteristics described in this paper. Our data indicate that 53% of the XLA D sequences involved D-D fusions using up to four germline D segments. The way they originate through multiple D fusions seems a general process of generating diversity in the D region, both in XLA and normal B cells (58). The reference based on 10% of D-D fusion for the adult PBL suggested by Yamada et al. (44) is in fact misleading for comparison, since these authors did not take inverted and DIR sequences into account in their conclusions.

A large variety of D segments was identified, including DIR genes, either in direct or reverse orientation. In fact, as it can be seen in table II, no particular pattern emerges in the D usage, which grossly reflects the estimated gene number. In this regard, the largest DXP family is also the most represented in XLA, but also in other physiological situations (58). By contrast, we did not observe the increased expression of DHQ52 that was reported in fetal liver (58). Finally, analysis of the reading frame usage revealed that all three frames were used, eventhough RF2 was found in a twofold excess. This observation is in sharp contrast with the situation depicted in the mouse, in which RF1 is largely overused, as the result of a strong selection process (59).

JH gene usage also reveals that the expression pattern of the XLA repertoire is close to that of the fetal liver (Fig. 8), with a major contribution of JH3 (which is only weakly represented in the adult repertoire), JH4 and JH6. Contribution of JH genes could vary in length, due to various truncations, especially for JH6, in agreement with previous reports (44).

N diversity (with possible examples of P diversity, see fig. 3) occurred on both V-D and D-J sides, and remained very limited, bringing another criteria of earliness (60, 61). It should be noted however that assignment of N regions remains partially questionable, in that they are defined in a negative way, from the absence of known germline gene counterpart. Undefined D genes may therefore lead to overweight contribution of N diversity.

Finally, random somatic mutations were present in limited extent on VH, D and JH, as can be expected for an early repertoire (60).

Thus, by all the analyzed criteria, the XLA repertoire resembles that of the normal fetal lymphopoietic tissues, suggestive of a typical pre-B phenotype. These results are in agreement with previous phenotypic characterization of the bone marrow cell population in XLA patients (62, 63). By contrast to other reports (3, 9, 49, 64), our data indicate that rearrangement mechanisms seem fully functional, although a possible decrease in efficiency of the pro-B to pre-B transition, as followed by the DJ to VDJ rearrangement cannot be quantitatively as-

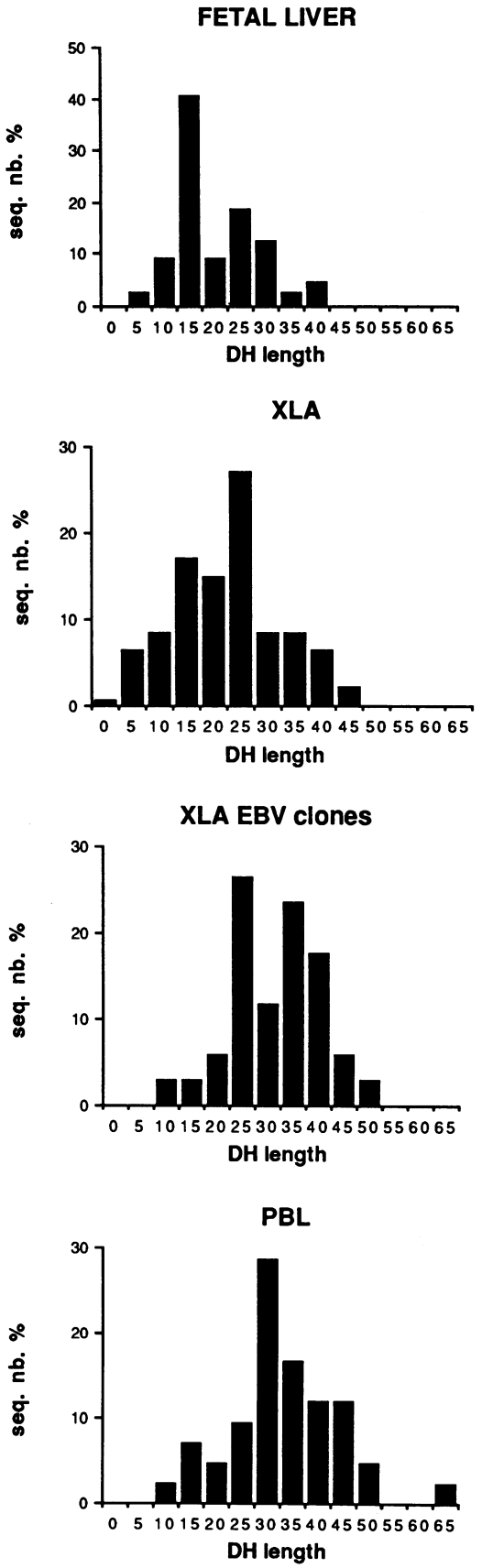

Figure 7. Length pattern of the D usage in XLA bone marrow cell transcripts (this work) compared to fetal liver (52), XLA-derived EBV clones (11–13), and normal adult PBL (44). Computed sequences cover N-D-N regions.

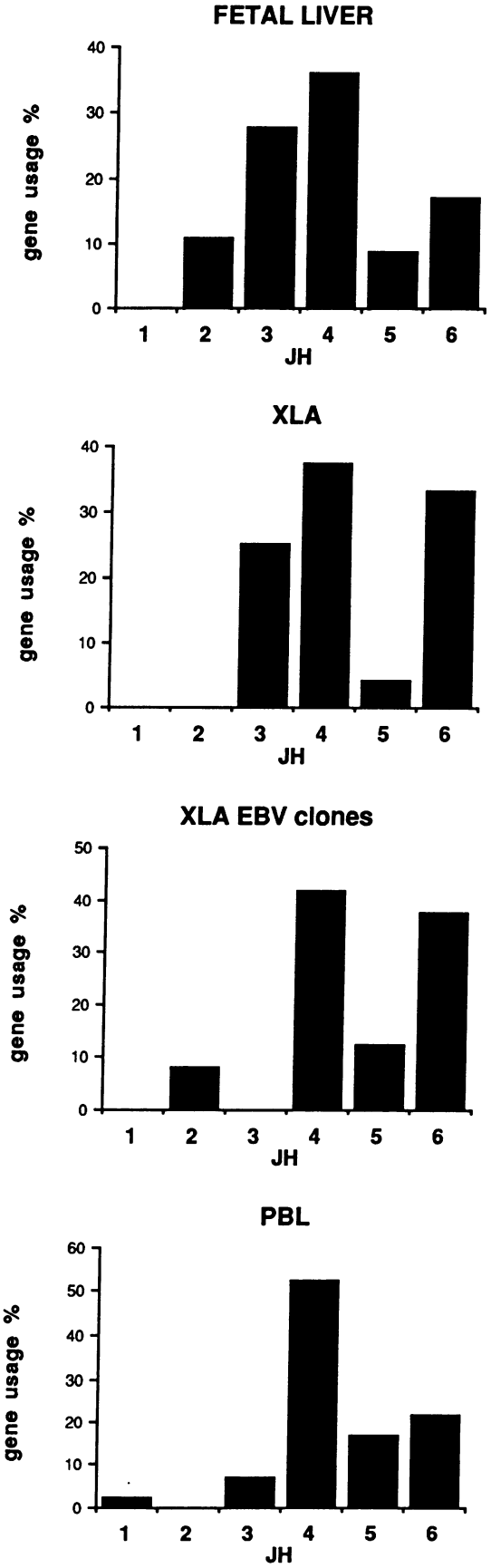

Figure 8. JH gene usage in XLA bone marrow cell transcripts (this work) compared to fetal liver (52), XLA-derived EBV clones (11–13), and normal adult PBL (44).

sessed from our data. These conflicting results might be reconciled by considering the XLA defect as a pleiotropic mechanism leading to a decrease in the efficiency of discrete steps of the early development of the B lineage, thus resulting in different phenotypes based on the severity of the disease. In addition, it can also be stressed that 88% of the transcripts had an open reading frame, imposing evidence that a selective process must operate at the pre-B stage. As the μ chains are associated with λ-like and V Pre-B gene products, the μ-ψL chain complex may already be actively involved in the acquisition and expansion of a pre-B specific repertoire that may condition the shaping of the mature HL receptors. In XLA cells, all steps leading to the shaping of the pre-B repertoire appear therefore functional. Preferential localization of the expressed VH genes to the most 3′ portion of the IGVH locus still raises the possibility that extension of the VH repertoire to the entire locus remains partially hindered. This may be in line with recent reports that discrete regulation of the 3′ vs 5′ VH usage operates in the mouse: (*a*) in mice transgenic for a human μ gene, endogenous V-D-J rearrangement was blocked except for the most 3′ VH (65) and (*b*) conversely, in Xid mice (66), another X-linked defect, preferential usage of the most 3′ VH is altered in ontogeny.

Our analysis performed on XLA bone marrow cells provides direct access to the pre-B cell repertoire which is truly representative of the defect. Analysis of EBV clones derived from XLA patients may therefore contain a bias linked to the selection of a minor population of B cells that have escaped the XLA defect (11, 12). The leakiness of XLA has been previously substantiated, and may be more or less important, in conjunction with the severity of the disease (7, 67, 68). In this context, the presence of κ transcripts in noticeable amounts in our samples indicate that some degree of leakiness has indeed taken place allowing the system a limited progression in the B cell differentiation pathway.

Acknowledgments

We express our deep gratitude to Dr. Honjo and colleagues for making available to us their manuscript in press in *Nature Genetics*. We also thank Drs. Cécile Tonnelle and Anne-Marie Cuisinier (Centre d'Immunologie de Marseille-Luminy [CIML]) for making their preprint available and for fruitful discussions. We also acknowledge expert technical assistance of Mrs. Elise Termine (CIML) and generous gift of DNA probes from Drs. F. Alt, J. Berman (University of Maryland, Baltimore), T. Honjo, M.-P. Lefranc (University of Montpellier, France), and P. Leder (Harvard Medical School, Boston).

This work was supported by CNRS, INSERM, Ministère de la Recherche et de l'Espace, and Association de la Recherche contre le Cancer.

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