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Research Article

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Premature Termination of Variable Gene Rearrangement in B Lymphocytes from X-linked Agammaglobulinemia

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Abstract

X-linked agammaglobulinemia (XLA) results from failure of B lymphocyte development. Immature B cells from a patient with XLA were found to produce truncated μ and δ immunoglobulin H chains encoded by D-J_H-C($\mu\delta$). The 5′ terminal sequence of cDNA encoding the H chains is composed of D-J_H with the characteristic GGTTTGAAG/CACTGTG consensus sequence utilized for V_H gene rearrangement upstream, and a leader sequence that serves for translation of this intermediate stage of rearrangement. Failure of variable region gene rearrangement may underlie the failure of B lymphoid development in XLA.

Introduction

The creation of functional antibody heavy (H) chains in developing B lymphocytes results from the ordered, somatic rearrangement of variable (V), diversity (D), and joining (J) segments genes (1–4). The order of this rearrangement is D onto J, followed by V onto the formed D-J element (3, 5). Inherited defects in any of these steps would lead to termination of gene rearrangement, failure of isotype diversification, absence of antibody production, and possibly to truncation of B cell development. One candidate for a disease caused by such an inherited defect, X-linked agammaglobulinemia (XLA),[1] is a sex-linked antibody deficiency disease (6–9). XLA results from a failure of B lymphoid development either at the stage of pre-B cells (major form), or at the later stage of early B cells, limited to expression of μ- and δ-H chains (8, 10). We have shown that the pre-B cells from three patients with the major form of XLA produce a truncated μ-chain, lacking 300 nucleotides of 5′ terminal sequence (9), due to transcription of the H chain gene locus prior to D-J rearrangement (manuscript in preparation). We now report that B cells from a patient with the minor form of XLA produce μ- and δ-chains of reduced size due to the absence of V_H. H-chain proteins are reduced in size by 13,000 D, lacking 180 nucleotides of 5′ terminal sequence. The truncated H chains are encoded by D-J_H-C(μ/δ), with a 5′ flanking sequence which encodes a leader sequence and the characteristic GGTTTGAAG/CACTGTG consensus sequence separated by 12 nucleotides, necessary for V_H gene rearrangement 5′ of the D (2), analogous to the Dμ proteins produced by Abelson virus transformed pre-B cells (11). Unlike Abelson virus transformed pre-B cells producing Dμ (11, 12), we have no evidence for spontaneous V_H gene rearrangement. We suggest that failure of V_H gene rearrangement underlies this form of XLA.

Methods

The patient with the minor form of XLA, identified in earlier reports as 4-8, has circulating B lymphocytes, limited to expression of δ- and μ-H chains (8, 13). B cell lines from this patient are also limited to δ- and μ-H chains, with delayed expression of L chain (13, 14). The presence of immature B cells in peripheral circulation distinguishes this patient from the patients with the major form of XLA, who lack all B lymphoid cells more mature than pre-B cells (10). Further, B cells from this patient react with antiserum specific for V region antigen (the protein product of V, D, and J), while pre-B cells from three patients with the major form of XLA produce H chain which lacks reactivity with anti-V (9).

B cell lines were established and grown as previously described (13), with the substitution of the alpha modification of MEM as medium. Cells were cloned suspended in soft agarose (15). For metabolic labeling of Ig, 10^7 cells were washed twice with balanced salt solution, and cultured for 4 h in MEM without methionine with 100 μCi of [^{35}S]methionine/ml at 3×10^6 cells/ml. Supernatants were reserved and cell platelets were lysed in isotonic buffer with 0.5% NP-40. IgD and IgM were isolated by precipitation with first antibody followed by Staph A (IgGSorb). Since IgM and IgD specifically reacted with Staph A in the absence of first antibody, isotypes were identified by immune precipitation with second antibody. Rabbit antiserum to μ- or δ-H chain, absorbed to remove nonspecific and L chain reactivity, was added to culture supernatant or cell lysate, and goat antiserum to rabbit IgG was added at equivalence (16). Precipitated Igs were redissolved, reduced, and electrophoresed in 12.5% SDS acrylamide slab gels exactly as described (17). The bands from proteins precipitated with second antibody were blurred compared to the Staph A precipitations, due to the presence of excess unlabeled protein from the precipitating antibodies. Unlabeled molecular weight standards, reduced normal human IgG (gamma and L chains), reduced normal human IgM, and bovine serum albumin, and labeled μ- and L chains from the normal B cell line SMI 4 were electrophoresed with the samples.

RNA was isolated from frozen cell pellets by the guanidine thiocyanate method of Chirgwin et al. (18) and the RNA was enriched for polyA containing sequences in oligo dT (19). Northern blots were run as described (20) and primer extension analysis was as previously described (21). cDNA libraries were constructed by the RNAse H method of Gubler and Hoffman (22), without *Escherichia coli* DNA

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1. *Abbreviations used in this paper:* XLA, X-linked agammaglobulinemia.

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ligase in second strand synthesis. Because of secondary structure of μ- and δ mRNAs, it was necessary to denature the RNA before first strand synthesis, either with CH_3HgOH and/or heating to 70°C for 20 min. cDNA was ligated into lambda gt10 (23), and infected into C600HflA.

Libraries were screened for μ-chain with the pμHM2 clone (9). δ-chain screening was with two 20-mer oligonucleotides encoded in Cδ. The 20-mer were used to isolate clone δ11 from the LAZ 166 library. This clone stopped 18 nucleotides into J. δ11 was used to isolate subsequent clones. μ-chain clones were subcloned into m13 mp18/19 and sequenced by Sanger dideoxy methods (24). Restriction mapping of the insert cDNA was performed as previously described (25), and fragments encoding Cμ were compared with the full length clone of a normal human μ-chain (26). Because of extensive secondary structure δ-chain clones could not be sequenced by chain termination. One δ-chain clone was subcloned into pUC 18, end labeled at either the Eco RI site at the 5′ end or the Asp 718 (Kpn I isoschizomer) site at nucleotide 97 of Cδ and the sequence determined by the method of Maxam and Gilbert (27). With few exceptions, the sequence was determined from both strands.

Results

We examined the sizes of δ- and μ-chains from cultures of Epstein-Barr virus transformed cell lines, derived from peripheral blood (LAZ 166) and bone marrow (SB25) of this XLA patient, and from two clones of LAZ 166, by SDS polyacrylamide gel electrophoresis. Southern blot analysis with probes to the J_H genes indicated that SB 25 was polyclonal while LAZ 166 had become monoclonal in culture. Clones C5 ($\mu+\delta+$ by immune fluorescence) and C6 ($\delta+\mu-$ by immune fluorescence) were isolated before LAZ 166 became monoclonal, and are different clonal representations of the patient's B cells. Precipitation of culture supernatants from the two cell lines with antiserum to μ- and δ-chains followed by Staph a yielded bands corresponding to full length L chains, truncated δ-chains of 49,000 D, and truncated μ-chains of 55,000 D (from SB 25) (Fig. 1). These sizes are compared to a calculated full-size of 63,000 D for δ-chain and a control full-size of 68,000 D for μ-chain. Since Staph A precipitation yielded both δ- and μ-bands from SB25, the isotype assignments were confirmed by precipitations with second antibody (16). When increased supernatant from LAZ 166 was precipitated with antiserum to μ-chain with second antibody, a band corresponding to the truncated μ chain of SB 25 was identified (Fig. 1). Because of the greater amount of δ-chain produced by LAZ 166, the truncated μ-chain could not be seen when both H chains were precipitated with Staph A. Immunoglobulins from two clones of LAZ 166, C5 and C6, were also examined. Clone C5 yielded a band corresponding to truncated μ-chain, while C6 yielded a band corresponding to truncated δ-chain (Fig. 1). A deletion of H chain mRNA was confirmed by Northern blot analysis, and found to be due to a 180 nucleotide deletion of 5′ terminal sequence by primer extension analysis (data not shown).

Full-length cDNA clones encoding μ- and δ-chains from LAZ 166 and cellular clones C5 and C6 were examined to determine the basis for the truncation. Clones μ16-4 and μ11-8 were isolated from the LAZ 166 parental cell line. Clone μ3A1A was isolated from cellular clone C5. Three δ-chain clones, δ4-2, δ3-1, and δ2-3, were isolated from cellular clone C6. Restriction mapping and sequence determination indicated that the deletion of the H chains was limited to the variable region of the μ and δ molecules. Restriction digestion

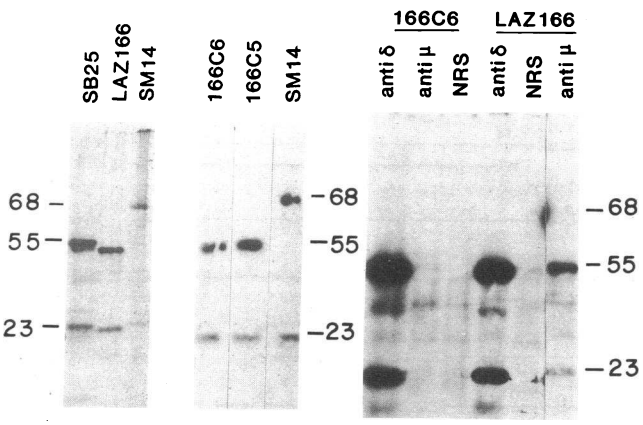

Figure 1. Truncated δ- and μ-chains from XLA B cells identified by SDS-acrylamide gel electrophoresis. Cell culture supernatants, metabolically labeled with [^{35}S]methionine, were immunologically precipitated, in the first instance, with antiserum to μ- and δ-H chains and Staph A, then electrophoresed in a 12.5% acrylamide slab gel. The second panel shows electrophoresis of specifically precipitated μ- and δ-chains: culture supernatants from clones C6 ($\delta+\mu-$) and C5 ($\delta+\mu+$) derived from LAZ 166 were immunologically precipitated with antiserum specific for δ(C6)- or μ(C5)-H chain with goat antibody to rabbit IgG at equivalence. The third panel demonstrates the specificity of the reagents used for precipitation and production of truncated μ-chain by the LAZ 166 parent cell line. Cell culture supernatants from clone C6 of LAZ 166 and LAZ 166 were incubated with antiserum specific for δ- or μ-H chain, or with normal rabbit serum (NRS), and then precipitated by the addition of goat antiserum to rabbit IgG at equivalence. Each of the lanes represents the product of 10^6 cells except for LAZ 166 precipitated with anti-μ and second antibody, where the product of 5×10^6 cells was used. Immunoglobulins from cellular lysates were of the same sizes. The positions of normal μ-, gamma, and L chains (68,000, 55,000, and 23,000 D, respectively), used as molecular weight markers, are denoted on the sides of the gels. Each panel is derived from a single slab gel. SMI4 is a normal B cell line which produces IgM. Broadening of the bands in the gels with second antibody precipitations is due to the increased protein necessary to make the complexes insoluble.

of the μ-chain clones with Eco RI yielded fragments of 840 base pairs (bp) and 637 bp, characteristic of full length Eco RI restriction fragments of the secretory form of Cμ (Fig. 2), and a fragment of 323 bp representing the 5′ terminal sequence, compared to 500 bp for full-length 5′ fragment from a normal μ-chain. Three δ-chain clones of full size were digested with Eco RI and Kpn I, yielding a 1300-bp fragment characteristic of full-length cDNA for the secretory form of Cδ and a 380-bp fragment consisting of the truncated variable region and the first 97 nucleotides of Cδ (Fig. 2). The 5′ fragment from normal, mature δ-chain is calculated to be 555 bp.

The 5′ terminal nucleotide sequence and the derived amino acid sequence of four cDNA clones is shown in Fig. 3. The sequences are composed of D, J_H, and Cμ or Cδ, without evidence for V_H region. The full 5′ sequence is shown for μ16-4 (isolated from the uncloned LAZ 166 cell line). Reading 3′ to 5′, Cμ is followed by a J region sequence which has a characteristic ATG found in J6. There are two single nucleotide changes from the published sequence of the most 3′ J gene, denoted J6 in reference 28, both of which conserve the derived amino acid sequence. J6 upstream of the ATG codon has been

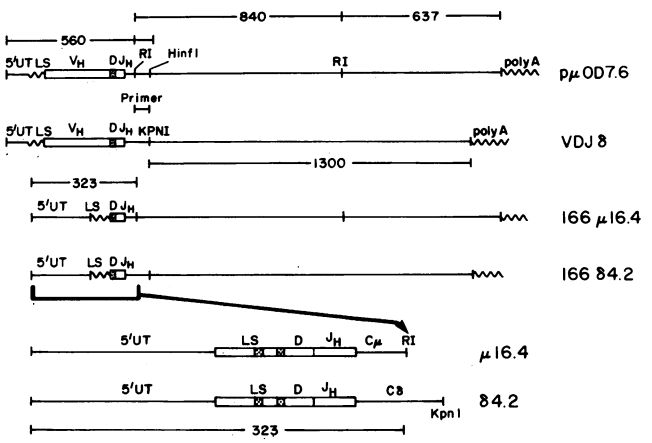

Figure 2. Restriction maps of cloned μ and δ cDNAs from XLA B cells. Shown at the top are normal, full-length mRNAs encoding μ- and δ-H chains. The sizes of restriction fragments for μ-chain were determined from cDNA of μ chain from the antibody to DNA, OD 7.6, derived from a normal B cell hybrid (26). δ-chain sizes were predicted from the 5' terminal structure of OD 7.6, and sequence analysis of Cδ from pδ11-1 cDNA clone from LAZ 166. Eco RI digestion of μ results in 3 fragments, of 560 nucleotides (5'), 840 nucleotides (middle), and 637 nucleotides (3' including only 20 nucleotides of the poly A tail). Cδ has no Eco RI sites, but a Kpn I site at nucleotide 97 of Cδ1 was used for subcloning. A 61 nucleotide restriction fragment resulting from Eco RI/Hinf I digestion of pμHM2 (shown below the pμOD7.6 restriction map) was used as primer for primer extension analyses, yielding a primed 5' extension fragment of 560 nucleotides from normal μ-chain mRNA. The cDNA clones μ16-4 and δ4-2 are shown. Both restriction fragments of Cμ resulting from Eco RI digestion are full-length, as is the Eco RI (linker added at 3' terminus for cloning) Kpn I fragment predicted for Cδ from the XLA B cell line. Below, the 5' terminal fragments of XLA B cell μ and δ are shown in expanded scale. Both isotypes are composed of a 5' untranslated sequence (5' UT), a proposed leader sequence (LS) which includes the conserved 9-12-7 acceptor sequence for rearrangement of V_H (2, 11), followed by D, J_H and Cμ or Cδ.

replaced by a sequence that has weak homology to published human D regions, and likely represents a previously unreported D region (28, 29). We were unable to determine what portion of the D-J interface may represent "N" region insertion (3). 31 nucleotides upstream of the ATG of J6 is the characteristic consensus sequence composed of a highly conserved 7-mer (CACTGTG) separated by 12 nucleotides from a 9-mer which is rich in A or T (2, 3, 28). The CACTGTG match is identical, while the 9-mer contains three Ts and two As. The 12 nucleotide separation sequence serves to distinguish the 5' terminus of D from that of J_H. An ATG codon in open reading frame with the D, J, and Cμ is located 69 nucleotides upstream of the consensus sequence-D interface. By analogy with the Dμ proteins described in mouse Abelson cell lines, the sequence between the ATG and the CACTGTG 7-mer may serve as a leader sequence (11, 30). Upstream of the ATG, there is an apparent 5' untranslated sequence consisting of 141 nucleotides.

Clone μ11-8, isolated from LAZ 166, likely represents the B allele of this monoclonal cell line. Like the μ16-4 A allele, it is composed of D-J_H-Cμ. An identical D region has been rearranged to a J region just upstream of the ATG codon. There is a single nucleotide substitution in J_H6 from μ11-8, likely a polymorphism carried on this individual's other allele. GTG (Val) is substituted for AGT (Ser) at the 5' end of the 12 nucleotide element of the 9-12-7 consensus sequence. An ATG codon is 69 nucleotides upstream of the consensus sequence-D junction, with the presumed downstream leader sequence differing from μ16-4 at 1 nucleotide.

The same D-J_H-C construction is present in δ-chain clone δ4-2, isolated from clone C6 of LAZ 166. Strong homology prevails through the 5' leader sequence, with divergence in the 3' and 5' ends of the D region, which is rearranged to a polymorphism of J_H3. This is followed by the sequence for Cδ. We have not determined the sequence of the other two δ-chain cDNA clones isolated from C6.

A D-J-Cμ cDNA resulting from nonproductive rearrangement was isolated from clone C5 of LAZ 166. The upstream leader sequence is very similar to, and the 9-12-7 acceptor site is identical to, the other sequences. In this clone a different D region has been employed, maintaining an upstream ATG in reading frame. However, a stop codon at position 23 would cause premature termination of translation. The stop codon is in a region most likely to represent "N" insertional sequence. J_H6 is again used with this sequence. Other μ-chain cDNA clones isolated from cellular clone C5 were incomplete.

Discussion

These data show that B cell lines from a patient with the minor form of XLA produce truncated δ- and μ-H chains. The truncated proteins are encoded by D-J-Cδ and D-J-Cμ, and likely result from termination of V_H-D-J_H rearrangement at this step. These D($\mu\delta$) transcripts are strongly analogous to the Dμ proteins described in mouse Abelson pre-B cells (11). We have not tested for the transcription of V gene elements prior to rearrangement described in Abelson cell lines (31). The 5' flanking sequences contain the characteristic 9-12-7 sequence thought to serve for rearrangement of V_H (2, 3, 11). There is an upstream ATG for initiation of translation, and the sequence that follows the ATG likely serves as a leader sequence, containing an N terminal basic region, a core of mostly nonpolar amino acids, and a more polar C terminal region (30). Three of the four D-J-C cDNAs are in open reading frame with the ATG and serve for productive translation of the truncated μ- and δ-proteins identified. The one nonproductive cDNA had a stop codon in the region likely to result from "N"region insertional material.

Abelson virus transformed pre-B cell lines producing Dμ have been reported to undergo spontaneous rearrangement of V_H, resulting in full length μ-chains (11, 12). In examining the two independently derived B cell lines from this patient, and multiple clones from LAZ 166, we have been unable to detect full length μ- or δ-chain as protein, RNA, or encoded in cDNA. The cDNA sequences represent D-J_H rearrangements and do not appear to result from abortive V_H gene rearrangement. V_H rearrangement would have eliminated the upstream 9-12-7 consensus sequences. The cDNA sequence upstream from the D region does not encode any of the several invariant residues of human V_H I, II, or III (26, 32). The most 5' ATG in the cDNA would initiate translation with the proposed leader sequence.

Reth and Alt (11) proposed that Dμ protein may have a role in regulation of V_H gene rearrangement. Demonstration

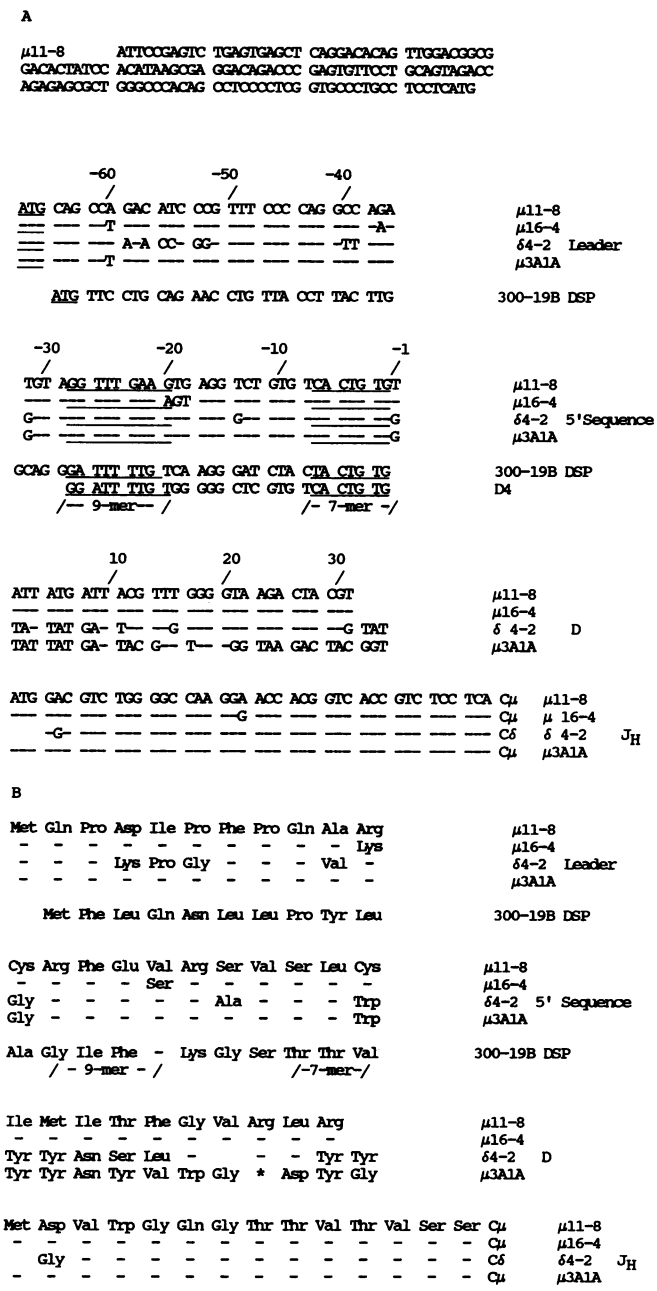

Figure 3. (*A*) Nucleotide sequences of the XLA B cell cDNAs encoding truncated μ- and δ-chains. μ11-8 and μ16-4 were isolated from the LAZ 166 parental cell line, which appeared monoclonal by J_H and kappa chain probes of Southern blots. δ4-2 was isolated from clone C6 of LAZ 166, and μ3A1A was isolated from clone C5 of LAZ 166. The entire 5′ sequence is given for clone μ11-8, which is used as a reference. Dashes in the other sequences indicate identity. There are 147 nucleotides of 5′ untranslated sequence, which includes the Eco RI linker added in cloning. Nucleotides are numbered from the 5′ terminus of the proposed D region, identified by the flanking sequence CACTGTG. The upstream proposed leader sequence begins with an underlined ATG sequence at nucleotide −67. The nonamer and septamer separated by 12 nucleotides required for V_H rearrangement are underlined and indicated. For the proposed coding region, the nucleotides are divided into codons, based upon the known reading frame of the J_H sequence. 300-19B DSP mouse Dμ 5′ flanking sequence (11) is shown in the same reading frame as the human 5′ flanking sequence. The 5′ flanking 9-12-7 sequence of a human D region, denoted D4, is also shown (28). There is a TAA stop codon beginning at nucleotide 24 of the μ3A1A sequence. (*B*) Polypeptide sequence of the 5′ terminal sequences. The amino acid sequence of the expressed D-J rearrangements was derived from the nucleotide sequences shown in A. The functional divisions of the polypeptides are shown.* The stop codon in the proposed N insertional region of D from μ3A1A.

of D($\mu\delta$) protein in human cells provides additional evidence for such a possible regulatory role. There is divergence between the upstream sequences of mouse and human D regions, in length of the 5′ untranslated region, and by inference in the location of the TATA box for initiation of transcription (we note that there is a TATA box-like sequence 280 nucleotides upstream from the human DHQ52 pseudo-D element [29]), and in the leader sequence. But the strong conservation of the upstream ATG codon and following leader sequence suggest a biological role for Dμ protein. A requirement for translation of Dμ protein would place additional restrictions upon phase of D rearrangement to J_H. Although a D element can be rearranged in all three reading frames (2, 3, 29), and especially with the possibility of N region insertion maintain the reading frame for translation of J-C (3), we have found only one ATG codon upstream of D. Additionally, the D regions we have described have only one translational reading frame without termination codons. Previously described D regions also carry termination codons in some alternate reading frames (28, 29, 32).

Premature termination of V-D-J rearrangement at D-J_H-C in XLA B cells could result from a failure of the enzymes which regulate V_H to D-J_H rearrangement. Alt and co-workers have proposed a common mechanism for rearrangement of the variable region genes of immunoglobulin H and L and the several T cell receptor variable regions (33). Consensus sequences (conserved nonamer-heptamer pairs separated by 12 or 23 nucleotides) at the 5′ or 3′ ends of the gene elements provide the structural basis for transient pairing of the gene elements (2). Yancopoulos et al. (33) have shown that a common recombinase acts in T and B cells, requiring hypothesized "specification elements" to determine the site of action of the recombinase, either by stabilizing the consensus sequence pairing or by providing access for the recombinase. Since T cell function in XLA is normal, the disease is unlikely to result from a defective recombinase. Alternatively, a failure of Ig V_H to D-J_H rearrangement could result from an altered specification element which either fails to catalyze recombination or causes abortive V_H gene rearrangement at another site. Defective rearrangement in the C.B-17 scid mouse model of severe combined immune deficiency usually resulted in elimination of the J elements in pre-B cells (34). We have not identified this form of abortive rearrangement in XLA.

The X chromosome linkage of this disease makes it unlikely that termination of rearrangement results from defective structural genes which are located on chromosome 14 (35). The D-J_H rearrangements identified in XLA B cells meet the known structural requirements for V_H gene rearrangement (2, 3). We suggest that there is a defect in the "specification elements" which regulate rearrangement of V_H genes to extant D-J_H. The H chains of these XLA B cells are distinct from the μ chains from pre-B cells of patients with the more common phenotype of XLA. Pre-B cells from three patients with the major phenotype of XLA lack V, D, and J region antigen, in

association with a 300 nucleotide deletion of 5′ terminal sequence (9), resulting from transcription of the H chain locus prior to D-J rearrangement (manuscript in preparation). The two phenotypes of XLA may result from mutations in closely related genes that regulate sequential steps in H chain rearrangement. XLA has been proposed to result from a central failure of B lymphoid development (36). The distinct blocks in H chain gene rearrangement could then be viewed as variable expression of this proposed central defect. Isolation of the gene that is defective in XLA may be required to resolve whether the failure of H chain rearrangement is primary to this disease.

B cell lines producing full-length, mature μ-chains have been established from rare B lymphocytes in the peripheral blood of patients with the major form of XLA (37, 38), indicating that the arrest of B cell development is not absolute. This finding is consistent with XLA resulting from failed regulation of B cell maturation, either of variable region rearrangement or from higher level regulation with a secondary affect on variable region rearrangement, rather than inherited defects in structural genes for Ig. We have not identified cells producing full length H chain from this minor form XLA patient. The absence of such cells may indicate that he has a mutation distinct from that of the major form XLA patients previously studied (37, 38), that his mutation is closer to absolute than the patients from whom such cell lines were established, or may be because his predominant, immature B cells producing D-J-C($\mu\delta$) are readily transformed by Epstein-Barr virus, in constrast to the pre-B cells of major form XLA patients.

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