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An aberrant splicing using a 3′ cryptic splice site within the C_H1 exon induces truncated μ-chain production

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SUMMARY

AT8–1-12–5-1, an Abelson virus-transformed immature B-cell line, produced truncated μ-chains. Sequencing analysis of the μ-expressed allele revealed that the variable region was an out-of-frame V_H7183–$D_{SP}2$–J_H3 complex. Two cDNA clones (5–1 cDNA1 and 5–1 cDNA2) derived from the transcripts of the μ-expressed allele were cloned and sequenced. Sequencing analysis of 5–1 cDNA1 revealed that the V_H7183–$D_{SP}2$–J_H3 sequence joined to the C_H1 exon at 136 bp, 3′ from the 5′ end of the C_H1 exon, resulting in the change of the reading frame from out-of-frame to in-frame. On the other hand, sequencing analysis of 5–1 cDNA2, which appeared to have derived from intron-containing premature mRNA, revealed that the J–C intron sequence joined to the C_H1 exon at 110 bp 3′ from the 5′ end of the C_H1 exon, indicating the deletion of 109 bp including the 3′ splice site of the C_H1 exon. These results demonstrate that the deletion of the authentic 3′ splice site of the C_H1 exon induced activation of the cryptic splice site within the C_H1 exon. This was followed by splicing of the variable region to the C_H1 exon at the cryptic splice site at 136 bp 3′ from the 5′ end of the C_H1 exon, resulting in the change of the reading frame from out-of-frame to in-frame, followed by the truncated μ-chain production.

INTRODUCTION

The RNA precursor of immunoglobulin heavy chains was transcribed after the correct recombination of V_H, D and J_H segment genes, and then RNA splicing processes removed the introns from the larger nuclear RNA precursor. Splicing of all eukaryotic genes, including immunoglobulin heavy chain genes, is mediated by the signal sequence 5′-GT-intron-AG-3′ at the junctions between introns and exons. Alteration of RNA splicing processes produces abnormal immunoglobulin heavy chains.

Production of truncated immunoglobulin heavy chains by the alteration of RNA splice sites has been reported elsewhere.[1–9] Deletion of the 3′ splice site of the C_H1 exon resulted in the complete deletion of the C_H1 exon, as frequently observed in heavy chain disease. Moreover, alteration of the authentic splice sites sometimes activated cryptic splice sites and induced an aberrant RNA splicing, resulting in abnormal immunoglobulin heavy chain production. Activation of the 5′ cryptic splice site within the V_H genes, followed by abnormal immunoglobulin production, has been reported elsewhere.[9–11]

We report here for the first time that production of truncated μ-chains in an Abelson virus-transformed immature B-cell line resulted from the activation of the 3′ cryptic splice site within the C_H1 exon, followed by the aberrant splicing of the variable region to the 3′ cryptic splice site.

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MATERIALS AND METHODS

Cells

AT8–1 and its subclones are Abelson virus-transformed immature B-cell lines established by *in vivo* infection of newborn BALB/c mice, as described previously.[12] Cells were cultured in RPMI-1640 medium containing 5% fetal bovine serum and 5×10^{-5} 2-mercaptoethanol. Cell cloning was performed in 0·33% agarose medium containing RPMI-1640 medium supplemented with 5% fetal bovine serum and 5×10^{-5} 2-mercaptoethanol.

Immunofluorescence

Detection of $\mu^+\gamma2b^+$ cells was performed as follows. The cells were fixed with acid–alcohol and then reacted with anti-mouse γ2b-chain antibodies (rabbit; Tago Inc., Burlingame,CA) at 37° for 30 min. After washing with phosphate-buffered saline (PBS), cells were stained with rhodamine-conjugated anti-rabbit IgG antibodies (Tago Inc.) plus fluorescein isothiocyanate (FITC)-conjugated anti-mouse μ-chain antibodies (N.L. Cappel Laboratories, Cochranville, PA), at 37° for 30 min, and examined by a fluorescent microscope.

Biosynthetic labelling and immunoprecipitation

Biosynthetic labelling and immunoprecipitation were carried

out as described previously.[13] Labelled immunoglobulins were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

Southern blot analysis

DNA was digested with *Eco*RI, electrophoresed through 0·7% agarose gels, blotted onto nitrocellulose filters, and hybridized to ^{32}P-labelled DNA probes. Hybridization was performed at 42° in 50% formamide, 5× saline sodium citrate (SSC), 1× Denhart's solution, 20 mM sodium phosphate (pH 6·5), 0·1% SDS, 10% dextran sulphate and 100 μg/ml sonicated denatured salmon sperm DNA. Filters were washed three times in 2× SSC and 0·1% SDS at room temperature, followed by washing twice in 0·1× SSC and 0·1% SDS at 65° for 20 min.

Immunoglobulin gene probes

The J_H region probe was a 1·5-kb *Hin*dIII/*Eco*RI fragment containing a J_H4 segment gene. The Cμ gene probe was a 1·2-kb *Hin*dIII fragment containing C_H3 and C_H4 domains.

Genomic cloning and DNA sequencing analysis

DNA was completely digested with *Eco*RI, size-fractionated by agarose gel electrophoresis, and recovered using a gene clean kit (Bio 101 Inc., Vista, CA). The fractions containing the rearranged J_H gene segments were identified by Southern blot hybridization using the ^{32}P-labelled J_H probe. The J_H-containing fractions were ligated into the bacteriophage vector λgt10 (Stratagene Corp., La Jolla, CA) and encapsulated using a λ-DNA *in vitro* packaging kit (Stratagene Corp.). Phage clones hybridizable with the J_H probe were then screened by plaque hybridization, and positively hybridizing plaques were identified on duplicate filters, picked out, rescreened, and grown in liquid culture. Appropriate restriction fragments were ligated into the PUC19 vector and sequenced by the dideoxy chain termination method with Sequenase (USB, Cleveland, OH), according to the manufacturer's instructions.

Northern blot analysis

Total cellular RNA was prepared by the guanidium thiocyanate/CsCl protocol. Poly(A)$^+$ RNA was purified through an oligo(dT)–cellulose column. Five micrograms of poly(A)$^+$ RNA was electrophoresed through a 1% agarose gel after denaturation with glyoxal and dimethylsulphoxide, and transferred to nitrocellulose filters. The filters were incubated at 42° for 16 hr with a ^{32}P-labelled DNA probe in reaction mixture containing 50% formamide, 5× SSC, 1× Denhardt's solution, 20 mM sodium phosphate (pH 6·5), 0·1% SDS, 10% dextran sulphate and 100 μg/ml denatured salmon sperm DNA. The filters were washed three times in 2× SSC and 0·1% SDS at room temperature and twice in 0·1× SSC and 0·1% SDS at 65° for 20 min.

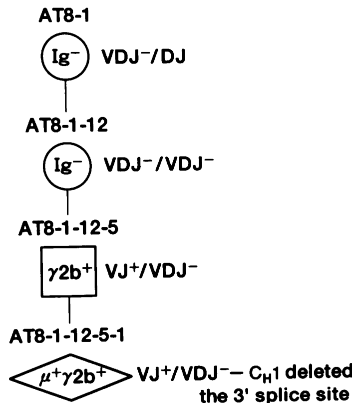


Figure 1. Schematic representation of the lineage of the various subclones. Ig, immunoglobulin.

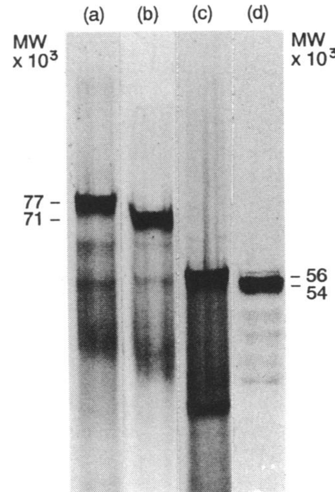


Figure 2. SDS–PAGE of IgH chains. Cell lysates were immunoprecipitated with mouse anti-μ (a, b) or anti-γ2b (c, d) antibodies. (a) PT3-2-1, an Abelson virus-transformed μ$^+$ preB-cell line; (b) and (d) AT8-1-12-5-1; (c) γ2b-producing hybridoma.

Construction of a cDNA library and sequencing analysis

A cDNA library was constructed using 5 μg of poly(A)$^+$ RNA from μ$^+$ γ2b$^+$ AT8-1-12-5-1 by using a cDNA synthesis kit

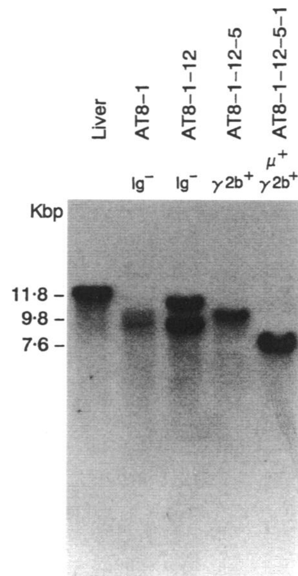


Figure 3. Southern blot analysis of μ$^+$ γ2b$^+$ AT8-1-12-5-1. DNA was digested with *Eco*RI and hybridized to the Cμ probe. AT8-1 showed broad bands because of the various deletions of the J–C intron during long-term culture.

(Pharmacia, Milwaukee, WI), according to the manufacturer's instruction. The cDNA library was screened with both the Cμ probe and the 0·6-kb *Pst*I fragment probe containing the V_HDJ_H complex of the μ-expressed allele of $\mu^+ \gamma 2b^+$ AT8–1-12-5-1. Appropriate restriction fragments were ligated into the PUC19 vector and sequenced by the dideoxy chain termination method with Sequenase.

RESULTS

Isolation of a μ,γ2b-double producer

Intracytoplasmic $\gamma 2b^+$ AT8–1-12–5 was generated from immunoglobulin-null AT8–1-12, which had a non-productive V_H36–60–$D_{SP}2$–J_H2 and a non-productive V_H7183–$D_{SP}2$–J_H3 complex, by joining the productive V_H36–60–$D_{SP}2$–J_H2 to germline J_H3, followed by class-switching from μ to γ2b.[13] Thus, $\gamma 2b^+$ AT8-1-12-5 had a productive V_H36–60–J_H3 and a non-productive V_H7183–$D_{SP}2$–J_H3 complex and produced 54 000 MW γ2b-chains without a D portion and shorter than normal-sized 56 000 MW γ2b-chains. Double staining of $\gamma 2b^+$ AT8-1-12-5 cells with anti-μ plus anti-γ2b antibodies detected about 0·2% intracytoplasmic μ, γ2b-double positive ($\mu^+ \gamma 2b^+$) cells, suggesting that these $\mu^+ \gamma 2b^+$ cells were generated from the $\gamma 2b^+$ cells during propagation in culture. To determine the mechanisms by which the $\mu^+ \gamma 2b^+$ cells were generated,

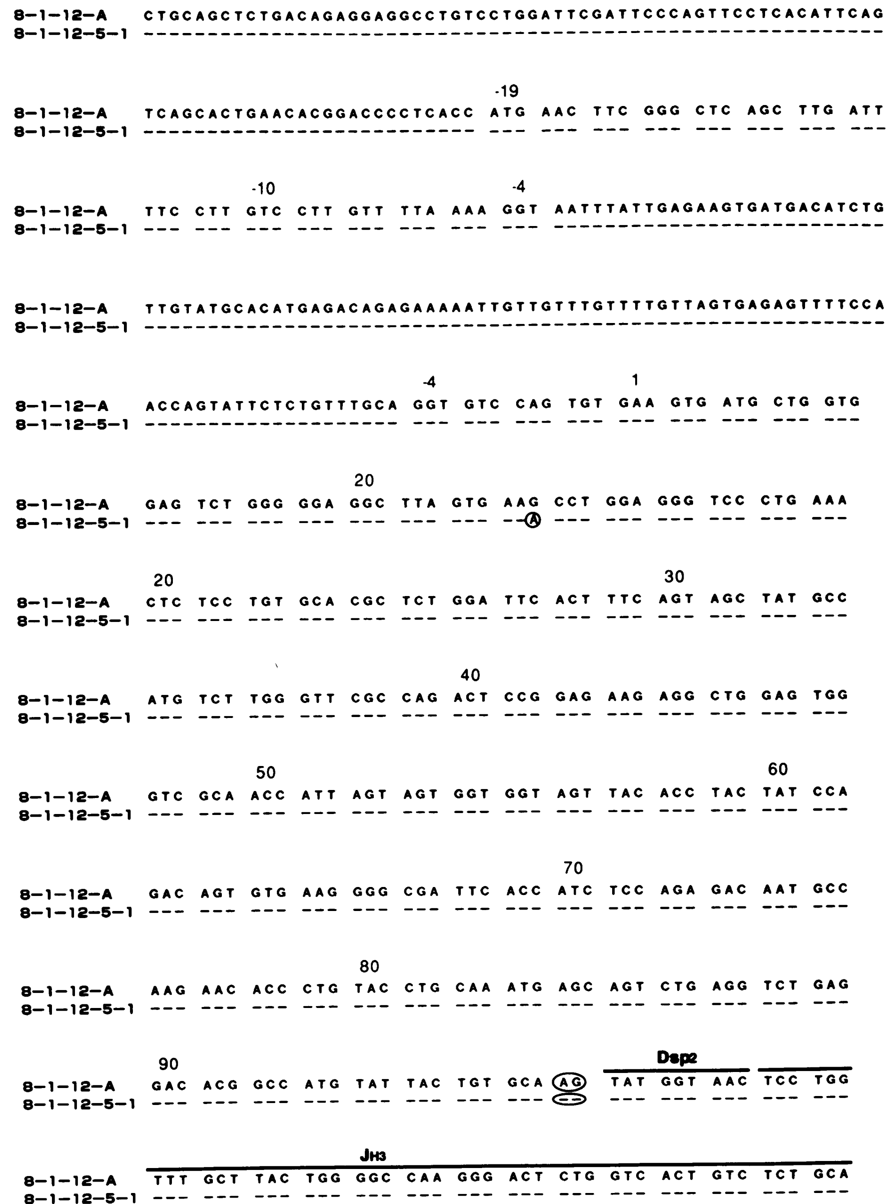


Figure 4. Sequencing analysis of the μ-expressed allele of $\mu^+ \gamma 2b^+$ AT8–1-12-5-1. 8–1-12-A represents a non-productive V_H7183–$D_{SP}2$–J_H3 complex of immunoglobulin-negative AT8–1-12.[13] A circle represents a point mutation. Ovals mark the position where the reading frames are not matched.

μ^+ γ2b$^+$ AT8–1-12–5-1, a subclone of γ2b$^+$ AT8–1-12–5, was isolated by cell cloning in soft agarose medium. In Fig. 1, the lineage of these subclones is shown, along with the salient characteristics of their rearranged immunoglobulin loci. To confirm the production of μ- and γ2b-chains in μ^+ γ2b$^+$ AT8–1-12–5-1, the cells were labelled with ^{35}S-methionine, and newly synthesized μ- and γ2b-chains were analysed by SDS–PAGE (Fig. 2). μ^+ γ2b$^+$ AT8–1-12–5-1 produced 71 000 MW μ-chains, shorter than the normal-sized 77 000 MW μ-chains, and 54 000 MW γ2b-chains.

Southern blot analysis

When DNA from μ^+ γ2b$^+$ AT8–1-12–5-1 was digested with *Eco*RI and hybridized to the J_H probe, it showed the same Southern pattern (two bands of 2·8 and 1·8 kb) as γ2b$^+$ AT8–1-12–5, as described previously.[13] When DNA was digested with *Eco*RI and hybridized to the Cμ probe, a 2·2-kb deletion of Cμ-associated *Eco*RI fragment was detected in μ^+ γ2b$^+$ AT8–1-12–5-1 compared with γ2b$^+$ AT8–1-12–5 (Fig. 3). Thus, the results suggested that the truncated μ-chain production in μ^+ γ2b$^+$ AT8–1-12–5-1 was induced by deletion of the Cμ-containing region.

Sequencing analysis of the μ-expressed allele

The V_HDJ_H complex of the μ-expressed allele of μ^+ γ2b$^+$ AT8–1-12–5-1 was cloned and sequenced (Fig. 4). The sequence was the same as that of γ2b$^+$ AT8–1-12–5[13] except for the presence of one point mutation, and the reading frame was out-of-frame.

Sequencing analysis of Cμ-containing cDNA clones

When Northern blot analysis of the RNA from μ^+ γ2b$^+$ AT8–1-12–5-1 was performed using the Cμ probe, two major bands of 2·2 and 2·0 kb were detected (Fig. 5). A 5·9-kb band detected at long exposure might have been premature mRNA. A cDNA library that was constructed from poly(A)$^+$ RNA of μ^+ γ2b$^+$ AT8–1-12–5-1 was screened by hybridization with the Cμ probe. Two-thirds of the Cμ$^+$ clones were hybridizable with the V_H probe. Five Cμ$^+$ V_H^+ clones and 11 Cμ$^+$ V_H^- clones were selected for further analysis. A Cμ$^+$ V_H^+ clone (5–1 cDNA1, 1·8 kb) and the largest Cμ$^+$ V_H^- clone (5–1 cDNA2, 3·4 kb) were sequenced (Fig. 6). Sequencing analysis of 5–1 cDNA1 revealed that the V_HDJ_H sequence joined to the C_H1 exon at

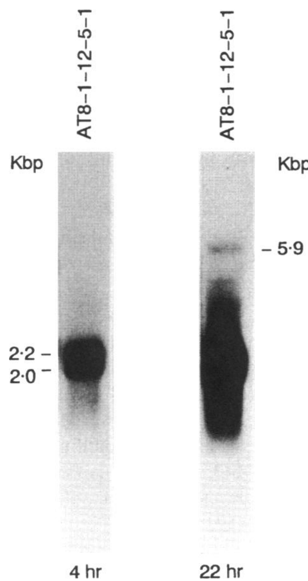


Figure 5. Northern blot analysis of μ^+ γ2b$^+$ AT8–1-12–5-1. Northern blots of 5 μg of poly(A)$^+$ RNA were hybridized to the Cμ probe. Autoradiographic film was exposed for 4 and 22 hr.

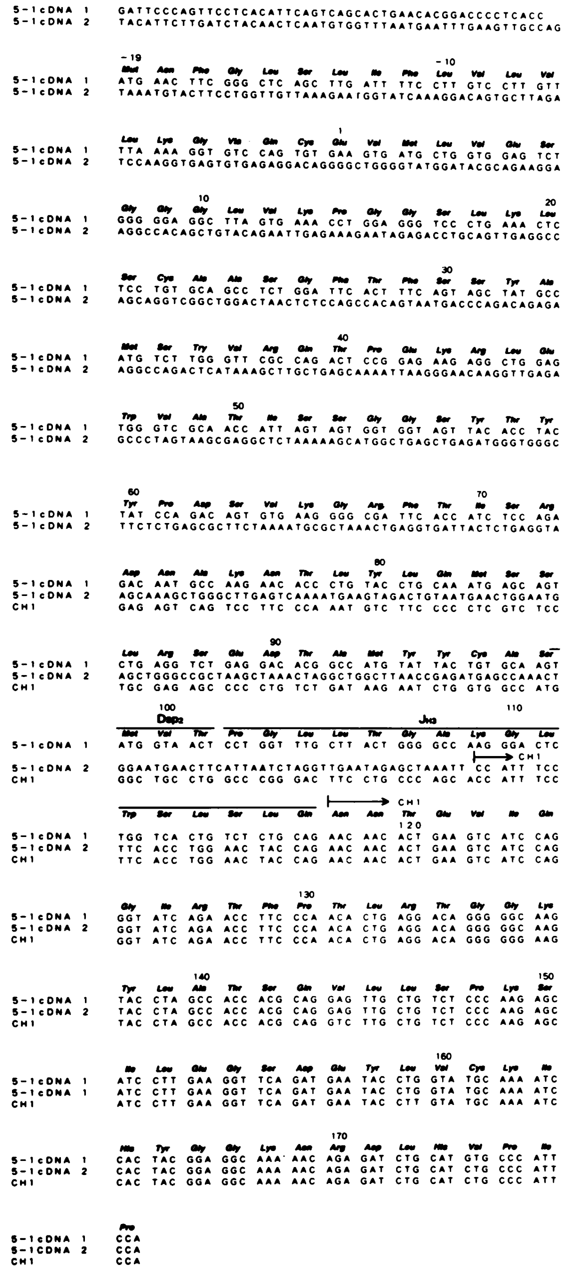


Figure 6. Sequencing analysis of cDNA clones derived from the transcripts of the μ-expressed allele of μ^+ γ2b$^+$ AT8–1-12–5-1. The nucleotide sequence of the C_H1 exon is described for comparison.

136 bp 3′ from the 5′ end of the C_H1 exon, resulting in the change of the reading frame from out-of-frame to in-frame. The V_HDJ_H sequence of 5-1 cDNA1 was the same as that of $\mu^+ \gamma2b^+$ AT8–1-12–5-1, indicating that the 5–1 cDNA1 was derived from the transcript of the μ-expressed allele of $\mu^+ \gamma2b^+$ AT8–1-12–5-1. On the other hand, sequencing analysis of 5–1 cDNA2 revealed that the J–C intron sequence joined to the C_H1 exon at 110 bp 3′ from the 5′ end of the C_H1 exon. Since 5–1 cDNA2 contained all of three introns of the $C\mu$ gene, it appears that the cDNA clone was derived from intron-containing premature mRNA of the μ-expressed allele (data not shown). These results indicated that the C_H1 exon was deleted by 109 bp 3′ from the 5′ end of the C_H1 exon, resulting in the loss of the 3′ splice site of the C_H1 exon, and that the V_HDJ_H sequence was directly spliced to the 3′ cryptic splice site within the C_H1 exon at 136 bp 3′ from the 5′ end of the C_H1 exon, resulting in the production of the truncated μ-chains.

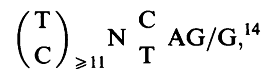
DISCUSSION

Sequencing analysis of the genomic DNA, and cDNA from the transcripts, of the μ-expressed allele of $\mu^+ \gamma2b^+$ AT8–1-12–5-1 showed that the C_H1 exon was deleted by 109 bp 3′ from the 5′ end of the C_H1 exon, resulting in the loss of the authentic 3′ splice site of the C_H1 exon. The deletion of the authentic 3′ splice site activated the 3′ cryptic splice site within the C_H1 exon, resulting in the splicing of the variable region to the cryptic splice site, followed by the production of truncated μ-chains.

It has been reported that the truncated μ-chain mRNA was created through the use of a cryptic splice donor site found within the V_H gene.[9–11] However, there are no reports on the activation of the cryptic splice site within the C_H1 exon. Thus, our present study is the first to report that activation of the cryptic splice site by the deletion of the authentic 3′ splice site induced the splicing of the variable region to the cryptic splice site within the C_H1 exon, resulting in truncated μ-chain production. Moreover, previous analysis of the truncated IgH chains in some cell lines, including the cell lines derived from heavy chain disease, demonstrated that the deletion of the 3′ splice site of the C_H1 exon resulted in the complete deletion of the C_H1 exon in all the cases.[6–8] The reason why only the cryptic splice site within the C_H1 exon was activated in our cell line, AT8–1-12–5-1, while other cell lines had deleted 3′ splice sites of the C_H1 exon is unknown. However, the extent of the deletion of the C_H1 exon might have an effect on the activation of the cryptic splice site.

The $V_H7183–D_{SP}2–J_H3$ complex of the truncated μ-expressed allele of $\mu^+ \gamma2b^+$ AT8–1-12–5-1 was out-of-frame. However, an aberrant splicing of the $V_H7183–D_{SP}2–J_H3$ sequence to the 3′ cryptic splice site within the C_H1 exon induced the production of truncated μ-chains, showing that the J_H3 sequence was translated by a reading frame different from the usual one. To our knowledge, this is the first report of an unusual translation of J_H sequences.

Since the sequence 5′-GCAG/A-C_H1-3′, found at the junction between the J_H3 and C_H1 exons in the 5–1 cDNA1, is similar to the consensus sequence of the 3′ splice site,



$$\binom{T}{C}_{\geqslant 11} N \; {}^{C}_{T} \; AG/G,^{14}$$

this sequence appears to have mediated the observed aberrant splicing.

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