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Research Article

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Presence of Immunoglobulin (Ig) M and IgG Double Isotype-bearing Cells and Defect of Switch Recombination in Hyper IgM Immunodeficiency

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Abstract

We established a transformed B cell line expressing both IgM and IgG on the cell surface from a patient with hyper IgM immunodeficiency using Epstein-Barr viruses. DNA and RNA of the cells were analyzed. DNA rearrangements of Ig J_H gene loci were observed on both chromosomes. Cloning and DNA sequence analyses showed that one has a $V_HD_HJ_H$ structure while the other has a D_HJ_H structure. Southern hybridization with 5'-S_μ and S_γ region-containing probes indicated germline configuration in the switch regions of μ and γ genes on both chromosomes. To test expression of μ and γ chains in the transformed cells at the mRNA level, we used the polymerase chain reaction with three kinds of synthetic oligonucleotides as primers, one of which was part of the V_H gene, while the other two were complementary to parts of C_μ and C_γ genes. Sequence analysis of the amplified products showed that the same $V_HD_HJ_H$ sequence is directly connected with either the C_μ or the C_γ sequence in the mRNAs. This is direct evidence showing that in double isotype-bearing cells one $V_HD_HJ_H$ exon in the transcript is alternatively spliced to C_μ or C_γ without DNA rearrangement. The defect in this disease could be at the S-S recombination stage. (*J. Clin. Invest.* 1990. 85:1722-1727.) immunoglobulin gene • double bearer • polymerase chain reaction

Introduction

Class switches of Ig heavy (H)[1] chain are mediated by DNA rearrangement between two characteristic repetitive sequences such as S_μ and S_γ in the case of a switch from IgM to IgG (1). Aside from the classical switch via S-S recombination, there have been reports of B cells expressing two isotypes such as IgM and IgG (2). In these instances, DNA rearrangements have not been detected in C_H gene loci (3-6). However, the presence of such cells and their biological roles in B cell differentiation are still controversial (7).

Hyper IgM immunodeficiency is a syndrome characterized by an elevated concentration of IgM and the absence of IgG and IgA in serum (8). Clinically, patients with this disorder are extremely susceptible to recurrent infections. *Pneumocystis carinii* pneumonia in early infancy and periodic neutropenia are frequent concomitants. Although this disease was originally described as X linked (9), other genetic patterns have also been reported, indicating clinical and genetic heterogeneity (10). A World Health Organization (WHO) scientific group (11) has reported that patients with this disease have normal numbers of IgM-bearing and IgM and IgD double-bearing B cells in peripheral blood, but no IgG- or IgA-bearing B cells, suggesting Ig isotype switching defect. In this study we established a B cell line expressing both IgM and IgG on the cell surface by transformation of peripheral blood cells from a patient with hyper IgM immunodeficiency using EBV. Analyses of the transformed cells indicate that in double isotype-bearing cells, one $V_HD_HJ_H$ exon in the transcript is alternatively spliced to C_μ or C_γ without DNA rearrangement.

Methods

Transformation of peripheral blood cells with EBV. Mononuclear cells were obtained from the peripheral blood of the patient by density gradient centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) (12). Five million mononuclear cells in 3 ml culture medium (RPMI 1640 [Gibco Laboratories, Grand Island, NY] supplemented with 10% heat-inactivated fetal calf serum [Bocknek Ltd., Rexdale, Toronto, Ontario, Canada], 2 mM L-glutamine, 10 mM Hepes, 1.9 mg/ml $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin) were mixed with 2 ml filtered supernatant of the B95-8 cell culture containing EBV (13) and incubated at 37°C for 24 h under 5% CO_2. Subsequently, cells were distributed to 96-well flat-bottomed microplates (Costar, Cambridge, MA) at a cell density of 1×10^3/well in culture medium. Peritoneal adhesive cells from a BDF_1 mouse were used as feeders. 3-4 wk after EBV infection, extensive cell growth was observed.

Immunostaining. After expansion of the cells for another 1-2 wk, the transformed cells were assayed for Ig expression by direct immunofluorescence as described previously (14). In brief, the cells were stained with fluorescein-conjugated goat $F(ab')_2$ anti-human γ chain-specific antiserum (Tago Inc., Burlingame, CA) (left, 100 times), or rhodamine-conjugated goat anti-human μ chain-specific anti-serum (Cappel Laboratories, Malvern, PA) (right, 100 times).

Southern blotting, cloning, and sequencing. Probes J_H, 5'-S_μ, and S_γ were prepared from the already published clones (15). Conditions of hybridizations were the same as described (16). Two rearranged clones, λHT112-1 and λHT112-2, were isolated from HT112 cell DNA by the ordinary cloning procedure (17). Nucleotide sequence was determined by the dideoxy method (18) after subcloning of the proper DNA fragments into Bluescript M13 vector (Stratagene, San Diego, CA) and introduction of serial deletion mutations (19).

Polymerase chain reaction (PCR). Four primers were chemically synthesized. The V_H primer is a 23-mer ACGGCTGTGTATTACTGTGCGAG, the μ primer is a 24-mer GAATTCTCACAGGAGACGAGGGG, and the first γ primer is a 21-mer AAGTAGTCCTTGACCAGGCAG. Since we could not discriminate which γ gene was expressed in HT112 cells by immunofluorescence, we used a sequence commonly observed among four C_γ genes (20) as a γ primer. After total RNA from HT112 cells was hybridized with either the μ or the first γ primer and incubated with reverse transcriptase, the V_H

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1. *Abbreviations used in this paper:* C, constant; D, diversity; H, heavy; J, joining; PCR, polymerase chain reaction; S, switch; V, variable.

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primer was added to each cocktail. PCR was carried out 25 times with Taq DNA polymerase (21). The size of the major amplified product was 170 bp in the μ primer–containing cocktail. Since several kinds of mRNAs were amplified in the γ primer–containing cocktail, we used another method to identify a γ message. First, cDNA was prepared from total RNA with the first γ primer. The single-stranded DNA products, the fractions longer than 80 nucleotides, which were separated by acrylamide gel electrophoresis, were pooled. The PCR was carried out with Taq DNA polymerase using the V_H primer and a second γ primer complementary to a location further upstream of the first γ primer. The second γ primer is a 17-mer GAAGACCGATGG-GCCCTT. The length of the major amplified product was 146 bp.

Results

Establishment of a double isotype–bearing cell from a hyper IgM immunodeficiency patient. The patient described in this paper was a 5-yr-old boy with typical clinical signs of hyper IgM immunodeficiency; that is, elevated IgM (309 mg/dl), decreased IgG (43 mg/dl) and IgA (11 mg/dl) in serum, and a past history of *P. carinii* pneumonia and periodic neutropenia. His younger brother had the same clinical signs and laboratory findings, suggesting an X-linked disease. In contrast to the description by the WHO scientific group (11), he had normal numbers of IgG- and IgA-bearing B cells in peripheral blood (i.e., surface IgG^+ cells, 2.8–3.1%; surface IgA^+ cells, 1.7–2.4%; surface IgM^+ cells, 5.2–9.4%; normal values in our laboratory were 3.7±1.6% [mean±SD] for IgG^+ cells, 2.2±0.8% for IgA^+ cells, and 8.8±3.2% for IgM^+ cells).

We established B cell lines by transforming the patient's mononuclear cells with EBV. Cell clones expressing IgM and IgG on their surfaces were selected. One of them (HT112) was studied further. After subcloning, 90% of the cloned HT112 cells expressed IgM on their surface and half of IgM-positive cells were IgG positive (Fig. 1).

Germline configuration in the Ig switch regions on both chromosomes. DNA was prepared from HT112 cells. We also prepared DNA from total mononuclear cells of the patient's peripheral blood as a germline control to avoid restriction polymorphism. The DNAs were digested with Hind III, and Southern hybridization was carried out with the J_H probe (Fig. 2 *a*) (22). In the HT112 cells' DNA, two bands were detected at 10.5 and 7.7 kb, as shown in Fig. 2 *b*. We cloned both bands and named λHT112-1 and λHT112-2. Several positive clones were isolated from each band. Restriction maps of the clones derived from the same band were identical, indicating clonality of HT112 cells. Although the size of the 10.5-kb band was apparently the same as that in germline DNA, both clones were rearranged ones. We determined the nucleotide sequences of rearranged regions in clone λHT112-1 (10.5-kb band) and clone λHT112-2 (7.7-kb band). As shown in Fig. 3,

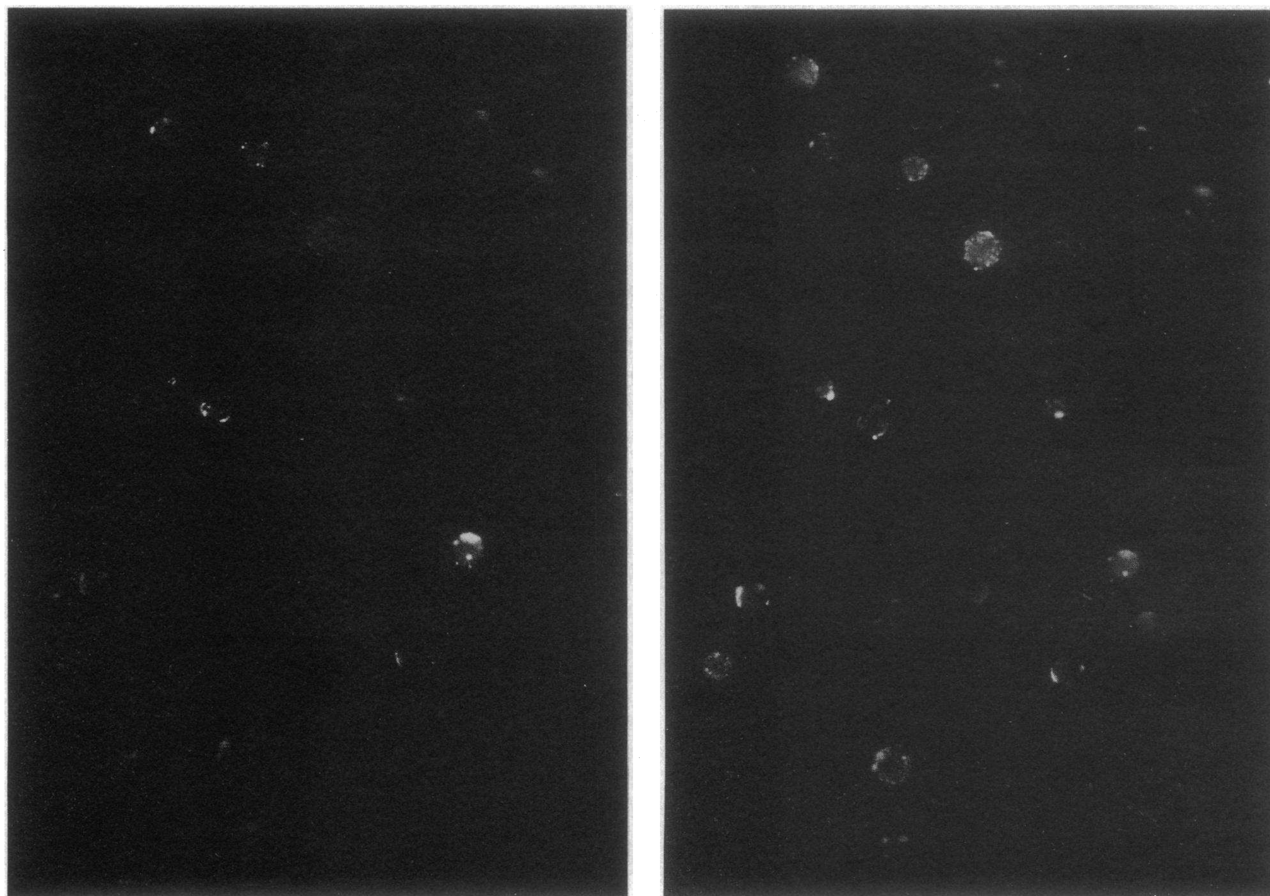

Figure 1. Simultaneous expression of IgM and IgG on the surface of HT112 cells established from a hyper IgM immunodeficiency patient. EBV-transformed HT112 cells were stained by FITC-conjugated anti–human γ chain (*left*) or tetramethyl-rhodamine isothiocyanate–conjugated anti–human μ chain (*right*) specific antiserum.

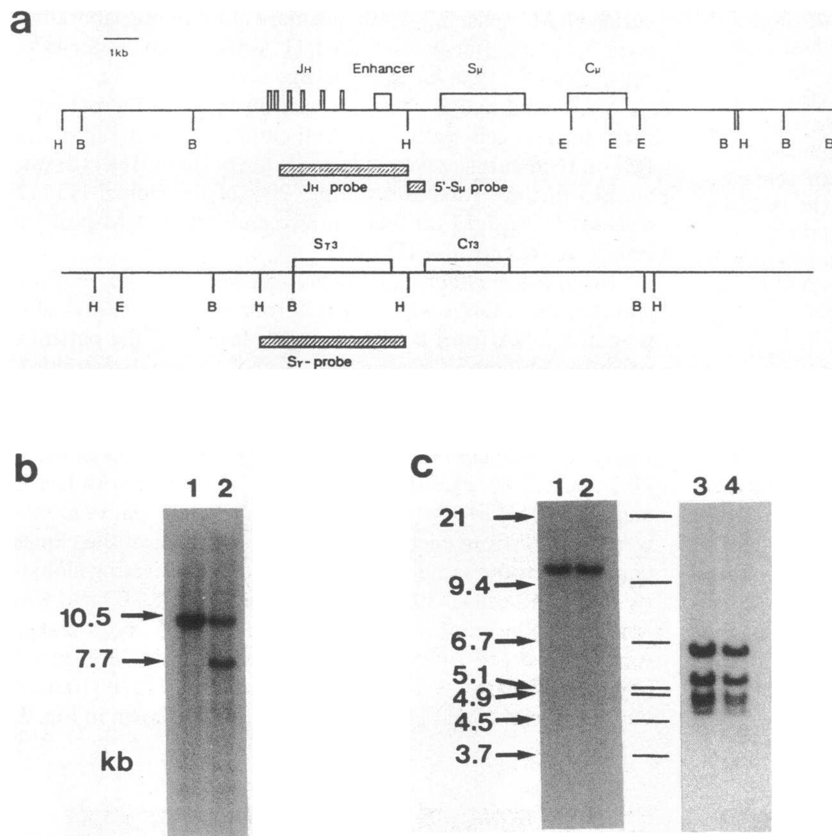

Figure 2. a, Restriction map of human IgH chain gene loci. Location of three probes, J_H, 5'-S_μ, and S_γ, are indicated. Restriction enzymes: *H,* Hind III; *B,* Bam HI; *E,* Eco RI. *b,* Southern hybridization of Hind III–digested DNA of germline (*1*) and HT112 cell (*2*) DNA with the J_H probe. *c,* Southern hybridization of Hind III–digested DNA of germline (*1, 3*) and HT112 cell (2, 4) DNAs with 5'-S_μ (*1, 2*) and S_γ (*3, 4*) probes.

a and *b,* the 10.5-kb band contains a complete $V_HD_HJ_H$ structure, and the 7.7-kb band contains an abortive D_HJ_H structure.

To examine whether the configuration in the C_H gene loci was germline or rearranged, two probes, 5'-S_μ and S_γ, were prepared (Fig. 2 *a*) (23). DNA rearrangements in four different S_γ regions can be identified by the single S_γ probe (24). In Hind III–digested HT112 DNA and total cells' DNA, Southern hybridization gave identical results (Fig. 2 *c*). Thus, in HT112 cells a $V_HD_HJ_H$ structure was formed on one chromosome and a D_HJ_H structure on the other chromosome, and no DNA rearrangements occurred in the switch regions on either chromosome.

One $V_HD_HJ_H$ sequence is directly connected with either C_μ or C_γ sequence in the transcripts. To test expression of μ and γ chains in HT112 cells at mRNA level, we adopted PCR (21) since we could not obtain enough material for Northern hybridization because of the low cell number. First, three kinds of oligonucleotides were chemically synthesized. V_H primer is part of the V_H gene, and μ and γ primers are complementary to parts of C_μ (24) and C_γ (20) genes, respectively. PCR was carried out as described in Methods. The size of the major amplified product was 170 bp in the μ primer–containing cocktail. Since several kinds of mRNAs were amplified in the γ primer–containing cocktail, γ message was amplified by two steps as described in Methods. The size of the major amplified products was 146 bp. Both 170- and 146-bp fragments were cloned and their nucleotide sequences were determined. As shown in Fig. 4 *a,* the same $V_HD_HJ_H$ sequence is directly connected with either C_μ or C_γ sequence. Judging from the sequence of C_γ region (20), either $C_{\gamma3}$ or $C_{\gamma4}$ was expressed in HT112 cells.

Discussion

In this study we established a transformed B cell line from the peripheral blood of a hyper IgM immunodeficiency patient using EBV. Membrane immunofluorescent analysis showed that approximately half of the cell population expresses both IgM and IgG. DNA analyses of Ig J_H gene loci and S regions indicated that the established cells are homogeneous and that the S regions are in the germline configuration. The presence of two kinds of mRNAs in which the same $V_HD_HJ_H$ sequence is connected with either C_μ or C_γ sequences was shown in these cells.

Several groups have already reported the presence of B cells expressing IgM and IgE (3) as well as IgM and IgG (4–6). The conclusions of these reports are basically the same as ours: the simultaneous expression of two different isotypes in a certain stage of B cell differentiation is mediated by alternative RNA splicing without DNA deletion. However, there are two strong arguments against this hypothesis. First, apparent expression of IgE and IgG molecules on the cell surface is due to expression of receptors for IgE and IgG, and to cytophilic association of IgE and IgG molecules (7). Second, in minor populations of B cells switching occurred from μ to other isotypes by DNA deletion, but Southern hybridization would not detect such deletion because of the low percentage of the switched cells (25). However, there are several reports showing that attaining the capacity to produce surface IgG does not preclude the potential for IgM expression (2). The reason it has been difficult to establish double-bearing cells in mice and humans is that they might be at a transient stage, and unstable in vitro.

In this study we established a double isotype–bearing cell

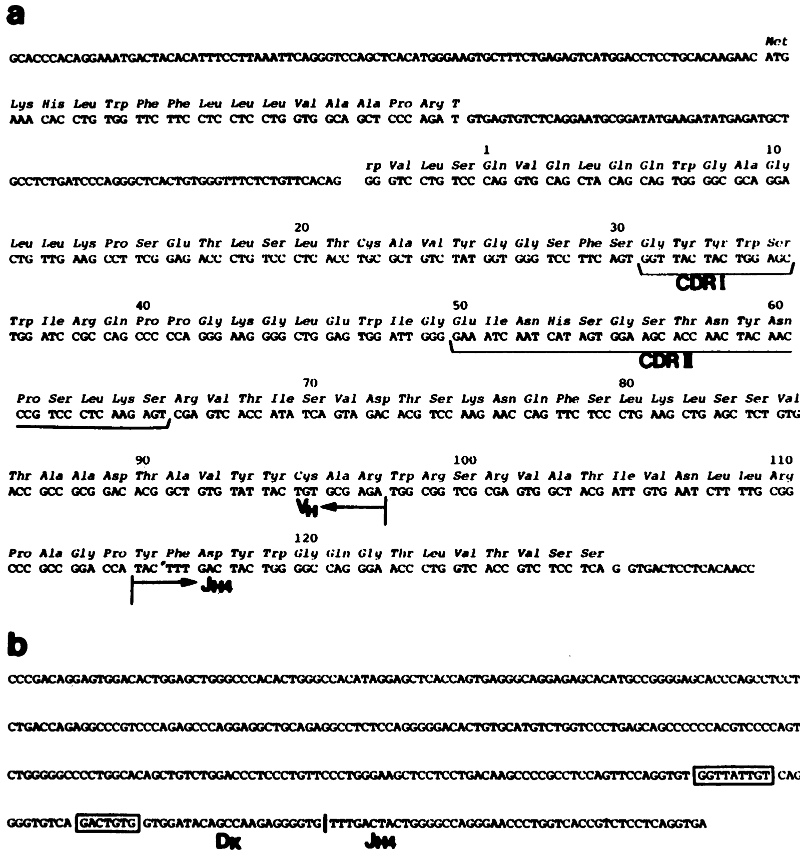

Figure 3. Nucleotide sequence of rearranged clones λHT112-1 and λHT112-2. *a*, λHT112-1 contains a complete $V_HD_HJ_H$ structure. *b*, λHT112-2 contains an abortive D_HJ_H structure. One D_K gene (33) was connected with the J_{H4} gene.

clone from the peripheral blood of a hyper IgM immunodeficiency patient. In this disorder, an isotype switch defect was proposed from the results of in vitro lymphocyte cultures, indicating either primary intrinsic B cell defects (26, 27) or secondary B cell defects due to "switch" T cell defects (28). Our case is uniquely suitable for studying double isotype–bearing cells because the patient has normal numbers of IgM^+ and IgG^+ B cells which failed to secrete IgG even when cocultured with healthy allogeneic helper T cells in the presence of pokeweed mitogen or *Staphylococcus aureus* Cowan I and recombinant IL-2 (data not shown). In a previous study (29) we established precursor B cell lines by transforming bone marrow cells from X-linked agammaglobulinemia and severe combined immunodeficiency patients with EBV. All the cells established from each patient showed similar phenotypes in both diseases. A defect in a gene involved in differentiation seems to cause an accumulation of a certain cell phenotype. In the present case of hyper IgM immunodeficiency, the ratio of IgG^+M^+/IgG^+ B cells was relatively high, 98±7% (mean±SD) in 13 assays, compared with the value in 9 healthy persons, 59±22% in our assays. Regardless of B or T cell defect, S-S recombination does not occur in this disorder, and double bearers exist in the B cell population of the patient. Our data could address the above criticism and provide stronger evidences than those in the previous reports (3–6). We directly showed that the same $V_HD_HJ_H$ sequence is connected with either C_μ or C_γ sequences in the mRNAs. This excluded the possibility that apparent expression of IgG molecules on the cell surface would be due to cytophilic association of IgG molecules. It is unlikely that the transcripts containing the $V_HD_HJ_H$ and C_γ sequences were products from switched cells with DNA deletion in minor populations of the established cell line for the following reasons. (*a*) The double bearer was established from a hyper IgM immunodeficiency patient. B cells in this patient do not seem to differentiate into IgG-secreting plasma cells because of a defect in S-S recombination. (*b*) IgG could not be detected on HT112 cell culture fluid by radioimmunoassay (14) or in cytoplasm by immunofluorescent technique (14) (data not shown). These results directly show that in double isotype–bearing cells, one $V_HD_HJ_H$ exon in primary transcripts is alternatively spliced to μ or γ without DNA rearrangement.

The biological role of IgM and IgG double-bearing cells in B cell differentiation is also controversial. We favor the idea that they are intermediates for class switch from IgM to IgG accompanied by DNA rearrangements. Recently, several groups (30–32) identified expression of germline C_H transcripts (so-called "sterile transcripts"), and proposed that expression of a certain isotype increases the accessibility of a putative class switch recombinase to the switch region, resulting in switch recombination. Apparently such sterile transcripts do not encode a protein. Lutzker et al. (31) argued that it seems unlikely that transcriptional events generating long transcripts from $V_HD_HJ_H$ to γ would target specific class switching in the context of an accessibility mechanism based on transcription, because intervening switch regions would also be transcribed. In a previous paper (15), however, we identified promoter activity in vitro upstream of C_γ genes. If some germline C_H transcripts are precursors for isotype expression by *trans*-RNA splicing (15), we do not have to assume such long primary transcripts. These double isotype–bearing

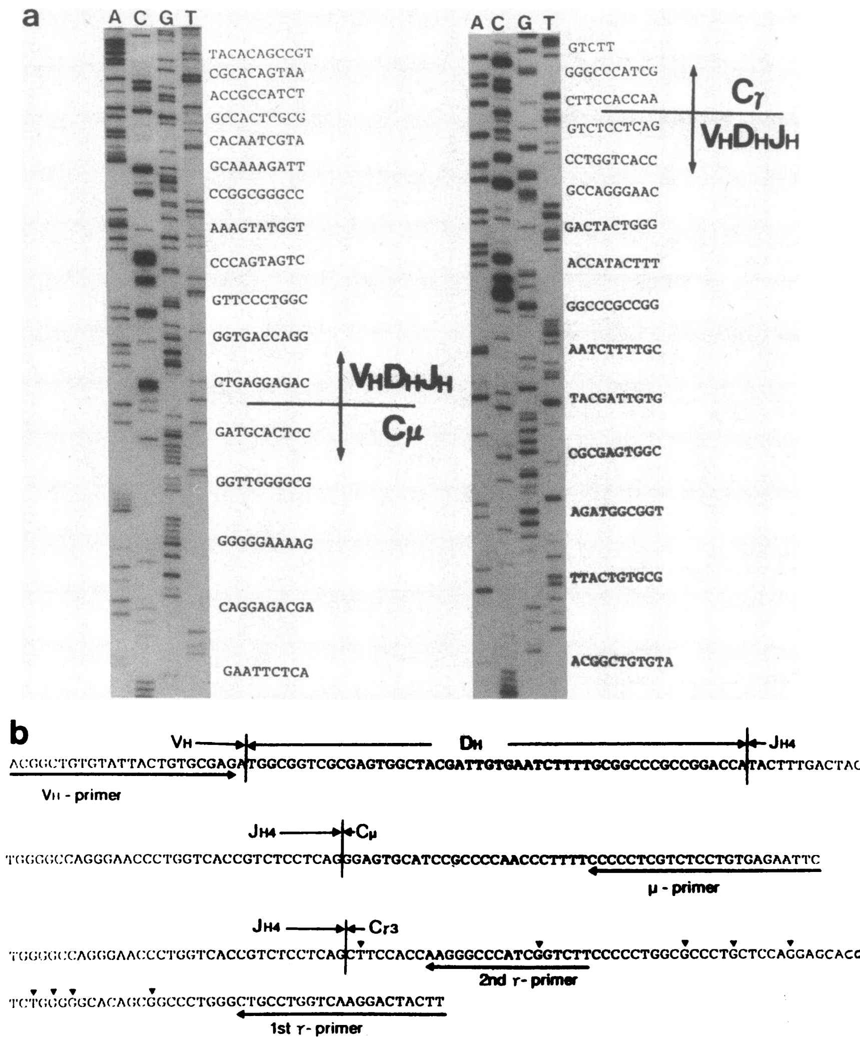

Figure 4. The same $V_HD_HJ_H$ gene is connected to either the C_μ or the C_γ gene. *a*, Sequence ladder $V_HD_HJ_H$-μ (*left*). Orientation of the sequence is from the μ to the V_H gene. *Right*, $V_HD_HJ_H$-γ. Orientation of the sequence is from the V_H to the γ gene. *b*, Nucleotide sequences around spliced regions. The sequence of the γ_3 gene is given. Triangles indicate the positions where different nucleotides are observed among four kinds of C_γ genes (20).

cells established in this study would be good systems to examine whether discontinuous transcription followed by *trans*-RNA splicing exists for γ expression in double producers.

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Mr. Akahori is a graduate student at Nagoya University.

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