Trans-Splicing as a Possible Molecular Mechanism for the Multiple Isotype Expression of the Immunoglobulin Gene

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Summary

We analyzed the molecular mechanism for the immunoglobulin (Ig) multiple isotype expression using a transgenic mouse (TG.SA) model system. Though most of the endogenous μ chain expression was excluded by the expression of the human rearranged μ transgene in the TG.SA mouse, a significant portion of splenic B lymphocytes could express the transgenic human IgM and endogenous mouse IgG simultaneously after stimulation with lipopolysaccharide and interleukin 4. The fluorescence-activated cell sorter®-purified population of the human IgM$^+$/mouse IgG$^+$ cells expressed mRNA that consisted of properly spliced sequences of the transgenic V_HDJ_H and the endogenous mouse C_γ genes (*trans*-mRNA), together with the transgenic human μ mRNA and germline transcripts of the mouse C_γ gene, without apparent rearrangement of the transgene. We also found that a lymphoma tumor, derived from the cross between the TG.SA mouse and another transgenic mouse carrying Ig H chain enhancer-driven *c-myc* oncogene, expressed about equal levels of the *trans*-mRNA and the transgenic μ mRNA without DNA rearrangement in either the transgene or the endogenous mouse switch region. These findings strongly support our previous proposal that the *trans*-splicing can account for the multiple isotype expression in this transgenic model and also suggest that novel molecular mechanism(s) might be involved in this reaction.

Progeny of a single B lymphocyte can switch the isotype of the expressed Ig from IgM to IgG or other classes without changing the variable region sequence that determines the antigen specificity (1, 2). This phenomenon, named Ig class switching, is mediated by DNA rearrangement of the constant region (C_H) genes of the Ig heavy chain. This DNA rearrangement, called S-S recombination, takes place between the S regions located 5′ to each C_H gene, except for the C_δ gene, and brings the V_HDJ_H exon to proximity of the C_H gene to be expressed by the deletion of the intervening C_H gene(s). Recent investigations have shown that the C_H gene(s) to be deleted by S-S recombination is excised out from the chromosome as a circular DNA (3–5). This finding provides the direct evidence for the intrachromosomal, looping-out deletion model for S-S recombination (6). These studies also indicate that IL-4 can modify the specificity of the recombination target gene (4, 5).

Although deletion of the C_H genes by S-S recombination occurs in myelomas (7, 8), hybridomas (9), and normal spleen cells (10), the deletion model has faced difficulty in explaining the observation that a single B lymphocyte can express more than one isotype simultaneously on the surface (11). From the experiments showing that sorted B cells (12, 13) or B cell tumors (14, 15) carrying two isotypes had no rearrangement of the expressed C_H genes, the involvement of RNA processing in multiple isotype expression was suggested, and such multiple isotype-expressing cells were proposed to be intermediates for class switching (2, 12). The production of multiple isotypes in a single B cell could be accomplished by either differential splicing of one continuous transcript of 200 kb or by intermolecular joining of two shorter transcripts (*trans*-splicing). Given the fact that specific germline transcripts were made from the target C_H gene(s) of S-S recombination (16–18), we proposed that such transcripts could be involved in the Ig multiple isotype expression as substrates (acceptors) for *trans*-splicing (19–21).

We have tested the *trans*-splicing model using a human μ chain transgenic mouse line (TG.SA) as a model system (20, 21). Despite the integration of the transgene outside of the mouse H chain loci, splenic B cells from TG.SA mice could synthesize a *trans*-mRNA that was generated by splicing the transgenic human V_HDJ_H exon sequence properly to the C_{H1}

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exon sequence of the endogenous mouse $C_{\gamma 1}$ gene. This *trans*-mRNA synthesis could be due to either S-S recombination between the transgene and the endogenous $C_{\gamma 1}$ gene or *trans*-splicing between their transcripts, but could not be accomplished by differential splicing of one contiguous transcript (20, 21).

To exclude that *trans*-mRNA was produced by interchromosomal S-S recombination, we have tested whether S-S recombination took place in the sorted B cells expressing transgenic human IgM and endogenous mouse IgG and a TG.SA-derived lymphoma tumor expressing the *trans*-mRNA. We found that these cells are expressing *trans*-mRNA without DNA rearrangement. Our data strongly suggest that the *trans*-mRNA is synthesized by the *trans*-splicing mechanism and that this mechanism may be involved in multiple isotype expression in B cells.

Materials and Methods

Transgenic Mouse and Cells Derived from it. The transgenic mouse line (TG.SA) carries a rearranged human V_HDJ_H-C_μ gene that allows expression of the membrane-bound μ chain alone (22). Pre- (no. 210) and pro- (no. 216) B cell lymphomas arose spontaneously by crossing the TG.SA line with another transgenic mouse carrying the Ig H chain enhancer-driven c-*myc* oncogene (23). The transgenic human μ mRNA was expressed in both tumors but the *trans*-mRNA was expressed in only no. 216 tumor. Hybridoma 2D9, which expressed endogenous mouse $\gamma 2$ transcript, was made by fusion between the spleen cell from TG.SA and the Ig-nonproducing myeloma NS1. TG.SA animals were maintained by crossing with FVB/N mice (Taconic Farms, Germantown, NY), or with C57BL/6 mice (SLC, Hamamatsu, Japan). The first generation of the animals crossed with C57BL/6 mouse were used for the sorting experiments.

Spleen Cell Culture, Flow Cytometry, and Sorting. Spleen cells from the TG.SA line were prepared and cultured 3 d in 5% CO_2-95% air at 37°C as described previously (20, 21). Fluorescence staining of surface Igs (20, 21) and brief acid treatment that dissociates Igs associated through Fc receptors (24, 25) were done as described. Stained cells were analyzed by flow cytometry on FACScan® and FACStar®, and human IgM^+/mouse IgG^+ cells were sorted using FACStar® by a Consort 30 program (Becton Dickinson & Co., Mountain View, CA). FITC-labeled anti-human μ chain antibody and R-PE-labeled anti-mouse γ chain antibody of goat were obtained from Southern Biotechnologies (Birmingham, AL).

Polymerase Chain Reaction. Cytoplasmic RNA from the sorted cells was extracted using ribonucleoside-vanadyl complex (New England Bio Labs, Beverly, MA) as an RNase inhibitor (26), and DNA from the same cells was extracted from the nuclear pellet at the same time. Total cellular RNAs from tumors and hybridomas were extracted by the guanidium isothiocyanate method (27). mRNAs were converted to cDNAs using oligo$(dT)_{12-18}$ primer and AMV reverse transcriptase (Seikagaku Kogyo, Tokyo, Japan; and Boehringer Mannheim Biochemicals, Indianapolis, IN) (20, 21). Kits for cDNA synthesis were purchased from Boehringer Mannheim Biochemicals and Amersham Japan (Tokyo, Japan). PCR was performed as described (20, 21, 28) using synthesized oligonucleotides as primers. Amplification was done by 50 cycles using an automated DNA thermal cycler and *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer/Cetus, Norwalk, CT). Primers for PCR were synthesized using automated DNA synthesizers (models 380A, B and 381A; Applied Biosystems, Inc., Foster City, CA). Positions of primers were as follows. Transgenic human V_H primer 1 (abbreviated as tV in figures), 24 mer from 356 bases upstream to the end of the J_{H6} in the V_H sequence, sense strand (20, 21, 29); transgenic human V_H primer 2 (tV-2), 21 mer from 332 bases upstream to the end of the J_{H6} in the V_H sequence, sense strand (20, 21, 29); human C_μ primer (hμ), 31 mer from position 31 to 1 of the C_{H1} exon, antisense strand (30); mouse $C_{\gamma 1}$ primer 1 (mγ), 21 mer from position 41 to 21 of the C_{H1} exon, antisense strand (30); mouse $C_{\gamma 1}$ primer 2 (mγ1-2, 21 mer from position 251 to 231 of the C_{H1} exon, antisense strand (30); mouse $I_{\gamma 1}$ primer (mIγ1), 21 mer from 186 base upstream of the end of the $I_{\gamma 1}$ exon (31); human c-*myc* exon 1 primer (m1), 21 mer from 412 bases upstream of the end of the 1st exon of the human c-*myc* gene, sense strand (32); human c-*myc* exon 2 primer (m2), 21 mer from 55 bases downstream of the beginning of the second exon of the human c-*myc* gene, antisense strand (32). Analyses of amplified fragments by agarose gel electrophoresis and nucleotide sequencing by dideoxy chain termination method (33) were done as described before (20, 21).

Other Molecular Biological Analyses. Southern blot hybridization was done as described (34) using probes that were labeled with ^{32}P by random hexamer priming method (35). Filters were washed at 65°C in 0.3× SSC-0.1% SDS in Fig. 4 or in 0.1× SSC-0.1% SDS in Fig. 6. The 1.0-kb SphI-HindIII fragment (36, 37; probe in a Fig. 2) of the human Ig H chain enhancer (human enhancer fragment 1), the 1.4-kb fragment staring from 200 bp upstream to the BglII site (36, 37; probe b in Fig. 2) of the human Ig H chain enhancer (human enhancer fragment 2), a human μ chain cDNA clone (19; probe c in Fig. 2), the 10-kb EcoRI-HindIII fragment of mouse $S_{\gamma 1}$ region (38, 39), and the 6.6-kb EcoRI fragment of mouse $S_{\gamma 2b}$ region crosshybridizing with $S_{\gamma 2a}$ region under stringent washing condition (39, 40) were used as probes to detect switch recombination. The 0.5-kb HindIII-BamHI fragment of *Evi*-2 (41; unique copy) and the 0.6-kb promoter region fragment upstream to the BamHI site of the ribosomal RNA gene (42; multiple copy) were used for calibration of DNA amounts. Autoradiograms of DNA blots from the sorted samples were taken and analyzed using a Bio-image analyzer (BAS2000; Fuji Film, Tokyo, Japan). RNase protection assay was carried out as described previously (43). Hybrids were digested with mixture of RNases A (0.1 mg/ml), T1 (100 U/ml), and T2 (10 U/ml) at 37°C for 40 min, and then run on 6% polyacrylamide gel containing 7 M urea. α-[^{32}P]-Labeled UTP and dCTP were purchased from New England Nuclear (Boston, MA) and Amersham Japan (Tokyo, Japan). Restriction endonucleases, T7 RNA polymerase, and Klenow fragment of DNA polymerase I were purchased from Takara (Kyoto, Japan) and New England Bio Labs, and used according to their instructions.

Results

Purification of Human IgM^+/Mouse IgG^+ Cells from Stimulated Spleen Cells of the Transgenic Mouse TG.SA. As we reported previously (20, 21), a significant portion (3–8%) of the spleen cells from the TG.SA transgenic mouse expressed transgenic human IgM and the endogenous mouse IgG simultaneously on their surface after a 3–4 d culture with LPS and IL-4 (Fig. 1 *A*), although expression of the endogenous mouse μ chain was almost completely excluded (20–22). To exclude the possibility that these Ig molecules on the cell surface were synthesized by some other cells, and cytophilically associated

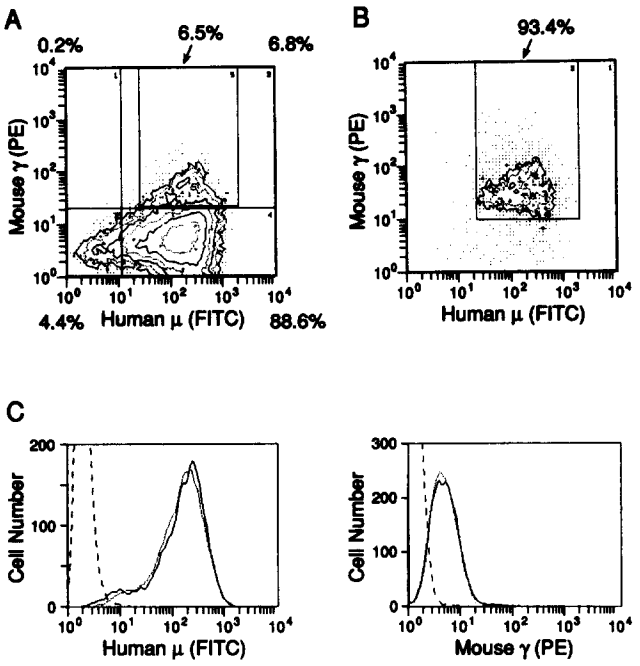


Figure 1. Detection of the human IgM$^+$/mouse IgG$^+$ double-positive cells in TG.SA spleen cells cultured with LPS and IL-4, and their purification by sorting. (*A*) Detection of double-positive cells. Spleen cells from TG.SA were cultured 3 d with LPS and IL-4, stained with FITC-labeled goat anti-human μ and PE-labeled goat anti-mouse γ, and analyzed by FACStar® during the sorting. 5×10^4 live cells were analyzed. The percentage of each quadrant is indicated at each corner and that of the target population is indicated at the top. (*B*) Purity of the sorted double-positive cells. The sorted cells were re-analyzed on FACStar® by the same setting as in *A*. 5×10^3 cells were analyzed. (*C*) Brief acid treatment to dissociate Igs bound to the cell surface through Fc receptors. TG.SA spleen cells were cultured as in *A*, and treated 1 min with pH 4.0 buffer before staining. The cells were stained simultaneously with anti-human μ (FITC) and anti-mouse γ (PE), and analyzed on FACScan®. Staining profiles for anti-human μ and anti-mouse γ were separately shown at the left and right, respectively. 10^4 live cells were analyzed. Broken, solid, and dotted lines indicate unstained control, staining without acid treatment, and staining after acid treatment, respectively.

through Fc receptors, the cells were treated briefly with acid to dissociate the interactions between Igs and Fc receptors. The staining profile of acid-treated cells was almost identical to that of untreated cells (Fig. 1 *C*), indicating that almost all Ig molecules detected on their surface were synthesized de novo. Their de novo syntheses were further confirmed by detection of mRNAs (see below). For further characterization of these human IgM$^+$/mouse IgG$^+$ cells at molecular level, we purified them by cell sorting. Starting from $\sim 2 \times 10^7$ TG.SA spleen cells cultured for 3 d with LPS and IL-4, we obtained $\sim 3 \times 10^5$ human IgM$^+$/mouse IgG$^+$ cells. The purity of the sorted cells was >90% as assessed by observation under a fluorescence microscope (92 of 100 cells observed were positive for both isotypes) and re-analysis using FACS® (Fig. 1 *B*).

Expression of trans-mRNA and Absence of Transgene Rearrangement in the Sorted Human IgM$^+$/Mouse IgG$^+$ Cells. To confirm the expression of *trans*-mRNA consisting of human V_HDJ_H and murine mouse C_γ sequences in the sorted human IgM$^+$/mouse IgG$^+$ cells, we took the PCR strategy as schematically illustrated in Fig. 2. By each combination of the primers indicated, we detected a clear band of the expected size for the transgenic human μ mRNA, the *trans*-mRNA, and the germline Cγ1 transcript (Fig. 3). From semiquantitative experiments by decreasing the amount of cDNA subjected to PCR, we could estimate that each human IgM$^+$/mouse IgG$^+$ cell contains >100 and >10 molecules on average of the transgene mRNA and the *trans*-mRNA, respectively (data not shown). These results strongly support the fact that the human μ chain and the chimeric γ chain were synthesized de novo by the human IgM$^+$/mouse IgG$^+$ cells.

The TG.SA mice carried two copies of the rearranged human μ chain gene as the transgene at a single locus on a chromosome different from number 12 where the endogenous Ig H chain locus is located. To test whether the *trans*-mRNA was produced by interchromosomal S-S recombination be-

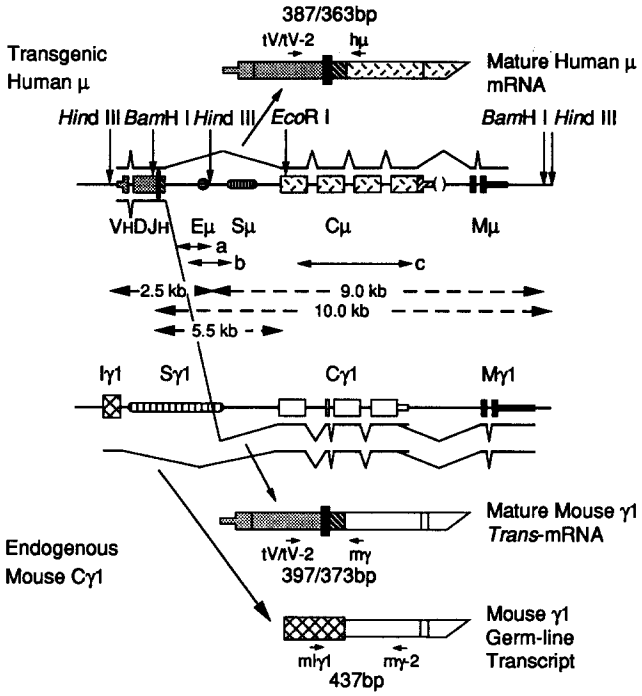


Figure 2. Schematic representation of the strategy to detect *trans*-mRNA by PCR and switch recombination of the transgene by Southern blot. Structures of the rearranged human μ transgene with only relevant restriction sites, the endogenous mouse $C_{\gamma 1}$ gene, a part of the transgenic μ chain mRNA, a part of the expected *trans*-mRNA, and a part of processed (spliced) form of the germline transcript of the $C_{\gamma 1}$ gene are schematically shown. Exons are indicated as boxes with different shading to distinguish functional regions. Enhancer and S regions are shown by circle and ellipses, respectively. A parenthesis in the transgenes indicates the deletion of the poly(A) addition site for the secretory μ chain. Positions of PCR primers are indicated by small arrows with abbreviated names, and expected sizes to be detected by PCR amplifications are shown above (transgenic μ) and below (*trans*-mRNA and germline transcript) the structures of the transcripts. Two expected sizes are given for different transgenic primers used (see details in Materials and Methods and Figs. 3 and 5). Positions of the probes used for the Southern blot are shown by solid arrows under the structure of the transgene, and sizes of the restriction fragments to be detected from the transgene without switch rearrangement are indicated with broken arrows.

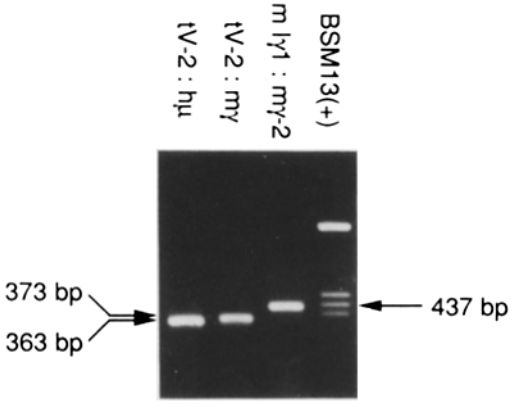


Figure 3. Detection of mRNAs in the sorted human IgM$^+$/mouse IgG$^+$ double bearers. Cytoplasmic crude RNAs from the sorted cells was converted to cDNA by reverse transcriptase. cDNAs equivalent to 5 ng RNA were subjected to PCR amplification for transgene mRNA and *trans*-mRNA detection, and 2.5 ng RNA-equivalent cDNA was used for detection of germline transcript. The products were analyzed by agarose gel electrophoresis. Primer combinations are shown above each lane. Bluescribe M13(+) DNA digested with HinfI was run at the right-most lane as size markers. Sizes of amplified fragments are shown by arrows.

tween the transgene and the endogenous C_γ gene, we analyzed the transgene configuration in the sorted human IgM$^+$/mouse IgG$^+$ cell population by Southern blot hybridization. At first a careful estimation of the relative amount of DNA from the sorted cells against the germline control DNA (liver or tail) was done either by ethidium bromide staining (Fig. 4 *A*) or by hybridization of the same filter with unrelated probes (Fig. 4 *B*). If the majority of the sorted cells underwent S-S recombination in one of the two copies of the transgene, the intensity of the transgene band in the double-positive cells should decrease to about half of that of the germline control, and the rearranged bands should migrate as a smear because of the heterogeneity of switch recombination sites. The relative intensities of the fragments (9.0-kb HindIII and 5.5-kb EcoRI+BamHI) encompassing the switch region of the transgene in the double-positive cells were almost equal to and more than 90% of those in the germline control DNA by densitometric analyses of the blots. The results indicate that the majority of human IgM$^+$/mouse IgG$^+$ cells did not undergo S-S recombination, and the simultaneous expression of transgenic human μ and chimeric mouse γ chains is best explained by the *trans*-splicing mechanism. It should be noted, however, that the conclusion based on the above results is limited because sorted IgM$^+$/IgG$^+$ cells may not be completely pure, and rearranged transgene, if any, in such heterogenous B cells cannot be detected as discrete bands.

Lymphoma from the TG.SA Line Expressed the trans-mRNA without S-S Recombination. To overcome the limitations of the above experiments, we looked for TG.SA-derived monoclonal B cell lines and tumors that express the *trans*-mRNA. The probe used for RNase protection assay should give rise to a 397-base protected band for the *trans*-mRNA between transgenic human V_HDJ_H and endogenous mouse C_γ ($C_{\gamma1}$, $C_{\gamma2b}$, and $C_{\gamma2a}$) genes, and a 356-base band for transgene products, because these C_γ genes have only a few discontinuous point mutations in the 5′ 41 bases (Fig. 5 *A*, *left*). 12 lymphomas derived from transgenic crosses between a lymphoma prone strain and a human IgM-carrying strain were screened (23). One, no. 216, a solitary pro-B cell lymphoma, that was negative for surface IgM but positive for the Ly5/B220 antigen and had no Ig gene rearrangements contained almost equal amounts of the *trans*-mRNA and transgene mRNA even under a stringent condition for the RNase treatment (Fig. 5 *A*). Under this RNase treatment condition using RNases A, T1, and T2, no artificially protected bands were observed in a mixture of human μ and mouse γ transcripts (Fig. 5 *A*).

To confirm that the 397-base protected product is due to the properly spliced *trans*-mRNA, we carried out the PCR experiment using the same primer combination as in our previous report (20, 21). The 3′ primer for the *trans*-mRNA

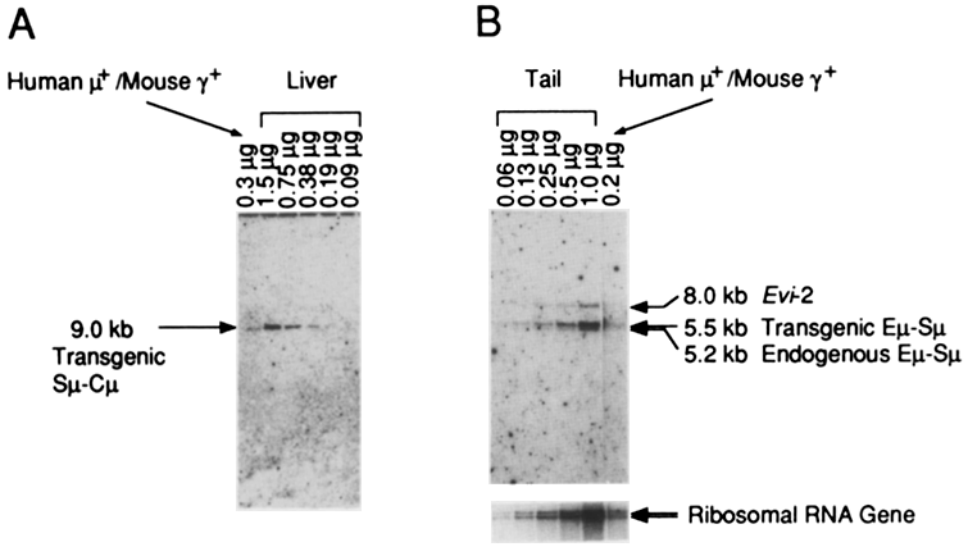


Figure 4. Absence of interchromosomal switch recombination in the sorted double bearers. Indicated amounts of DNA from the sorted cells and liver DNA from the same line (*A*) or tail DNA from the same animal (*B*) were digested with HindIII (*A*) or EcoRI plus BamHI (*B*) and subjected to electrophoresis in 0.6% agarose gel and transferred on to nylon (*A*) or nitrocellulose (*B*) filters. The filters were hybridized with human μ chain cDNA (probe c in Fig. 2) in *A*, or a mixture of the human enhancer fragment 1 (probe a in Fig. 2) and *Evi*-2 fragment in *B*. The filter used in *B* was rehybridized with ribosomal RNA gene probe to confirm the DNA amount, and the result is shown below *B*. Sizes of the detected bands are shown by arrows.

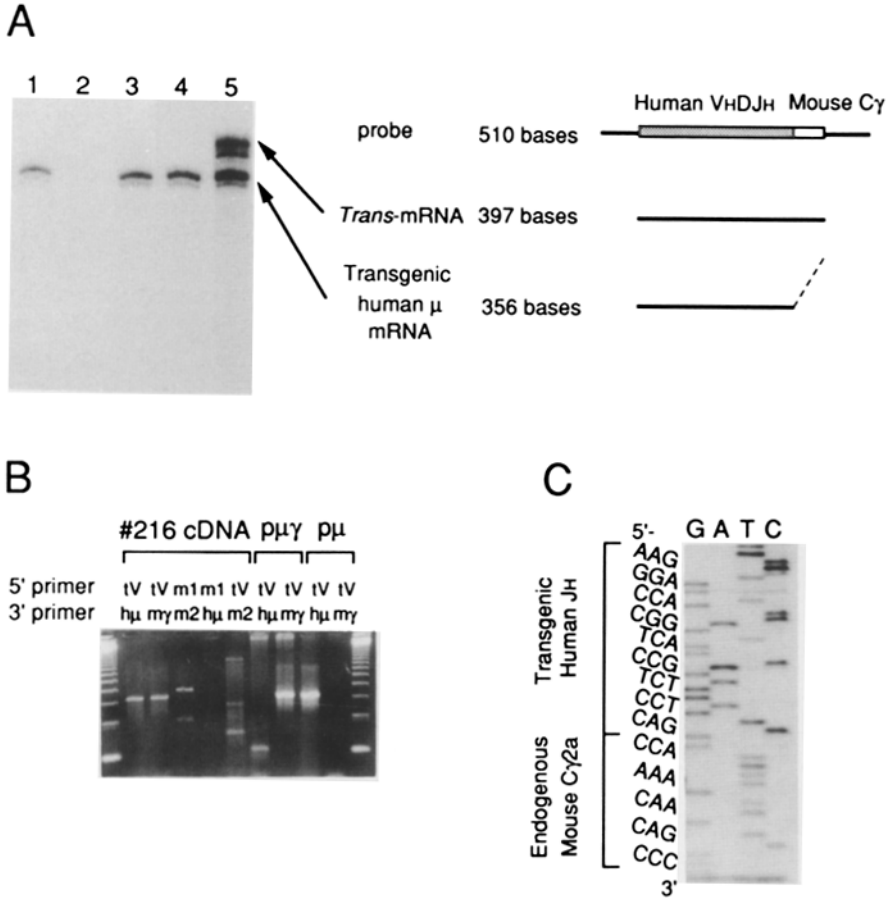
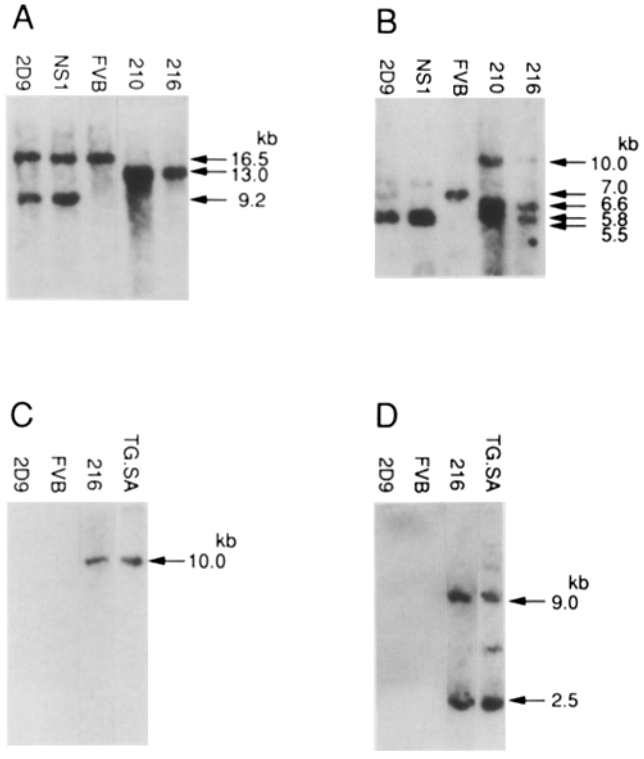


Figure 5. Characterization of *trans*-mRNA expressed in a pro-B cell tumor derived from the TG.SA line. (*A*) Detection of *trans*-mRNA by RNase protection assay. 10 μg each of total RNA from HeLa cell transfected by SV40-driven human μ (lane *1*), hybridoma 2D9 expressing γ2 transcript (lane *2*), the mixture of the former two RNAs (lane *3*), tumor no. 210 (lane *4*), and tumor no. 216 (lane *5*) were hybridized with *trans*-mRNA probe as shown at right. The small protected fragments (41 bases) expected from γ chain transcripts (lanes *2*, *3*, and *5*) were not detected in this hybridization and digestion condition. Sizes of protected fragments and their origins are shown. (*B*) Specificity of splicing combination checked by PCR. cDNA of tumor no. 216 and cloned cDNAs of *trans*-mRNA (pμγ) and transgenic μ mRNA (pμ) were subjected to PCR using primer combinations indicated above the gel. Ladders of multiples of 123-bp fragments were run as size markers at both sides. The expected sizes for the PCR products were: tV-hμ, 387 bp; tV-mγ, 397 bp; m1-m2, 467 bp; m1-hμ, 443 bp; tV-m2, 411 bp. Bands visible in tV-m2 lane of no. 216 cDNA and tV-hμ lane of pμγ were of wrong sizes. (*C*) Sequence analysis of PCR-amplified cDNA of *trans*-mRNA. The PCR-amplified fragment from tumor no. 216 cDNA using tV-mγ primer combination was subcloned and sequenced. Sequence ladders of the antisense strand at the J-C exon junction are shown with the read sequence of the sense strand at the left side. Exonal regions and their junction are also indicated.



detection could amplify $C_{\gamma 1}$, $C_{\gamma 2b}$, and $C_{\gamma 2a}$ mRNAs because it was complementary to the mouse $C_{\gamma 1}$ sequence with only one base difference from the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ sequences. DNA fragments of expected sizes for the transgenic mRNA and the *trans*-mRNA were amplified from tumor no. 216 cDNA (Fig. 5 *B*). Specificity of the primers was confirmed by amplification of previously cloned cDNAs of transgenic μ mRNA and *trans*-mRNA. The transgenic V_H primer and mouse C_γ primer combination gave no band from transgene cDNA clone (Fig. 5 *B*). Since no amplified bands were de-

Figure 6. Absence of interchromosomal switch recombination of the transgenic and endogenous switch regions in tumor no. 216. DNAs from hybridoma 2D9, its fused myeloma NS 1, FVB/N mouse liver, tail of TG.SA line, and tumors nos. 210 and 216 were digested with XbaI (*A* and *B*), BamHI (*C*), or HindIII (*D*), and separated by agarose gel electrophoresis followed by transfer to nitrocellulose filters. Filters were hybridized with mouse $S_{\gamma 1}$ (*A*), mouse $S_{\gamma 2b}$ (*B*), or human enhancer 2 (probe b in Fig. 2; *C* and *D*) probes. There were RFLPs in $S_{\gamma 1}$ and $S_{\gamma 2}$ regions among FVB/N and CD1 outbred mice in which the TG.SA line was established and maintained at an earlier stage. 2D9 hybridoma was made from the TG.SA animal maintained in FVB/N background, therefore, the control should be FVB/N liver and the fusion partner NS 1 DNAs. Control for tumor no. 216, which arose from the animal of CD1 background should be tumor no. 210, which arose from a sibling outbred animal and did not express the *trans*-mRNA.

tected by combination of transgenic V_H and c-*myc* oncogene primers, or c-*myc* oncogene and transgenic C_μ primers, in spite of the presence of abundant c-*myc* mRNA in this tumor, combination of the exon sequences spliced in *trans*-mRNA was specific. Amplified cDNA of the *trans*-mRNA from tumor no. 216 was subcloned and sequenced. As shown in Fig. 5 *C*, the transgenic V_HDJ_H exon sequence was properly spliced to the first exon sequence of the mouse $C_{\gamma 2a}$ gene. This sequence data unequivocally demonstrate that the tumor no. 216 expressed the properly spliced *trans*-mRNA of the transgenic V_HDJ_H and endogenous mouse $C_{\gamma 2a}$ regions, and also exclude the contamination of previously cloned cDNA (V_H-$C_{\gamma 1}$) because we have never constructed artificial *trans*-mRNA or cDNA of the γ2a isotype.

Monoclonal DNA rearrangement in the no. 216 lymphoma should be easily detected. We have analyzed rearrangement of the endogenous S regions for the $C_{\gamma 1}$, $C_{\gamma 2b}$, and $C_{\gamma 2a}$ genes and the transgene by Southern blot hybridization using mouse $S_{\gamma 1}$ (Fig. 6 *A*), $S_{\gamma 2b}$ (crosshybridized with $S_{\gamma 2a}$) (Fig. 6 *B*), and human Ig H chain enhancer regions (Fig. 6, *C* and *D*) as probes. None of these probes detected monoclonal rearrangement of the transgene and the C_γ genes. The relative band intensities of these genes remained unchanged, excluding polyclonal rearrangement as well. These results strongly suggest that no. 216 tumor cells expressed the *trans*-mRNA without S-S recombination and support the fact that *trans*-splicing is a likely mechanism for generation of *trans*-mRNA.

Discussion

We have shown that the *trans*-mRNA containing the transgenic human V region and the endogenous C_γ sequences are expressed in lymphoma cells as well as double-positive sorted spleen cells derived from the TG.SA transgenic mice. These cells did not show any sign of S-S recombination. The results support the hypothesis that *trans*-splicing is responsible for generation of *trans*-mRNA. Synthesis of *trans*-mRNA does not seem to be dependent on either the specific insertion site of the transgene or the specific V or C region sequence of the transgene, because we could detect *trans*-mRNA in similarly stimulated spleen cells of another transgenic mouse line that carried a rearranged mouse V_HDJ_H-C_μ gene (H. Han, A. Shimizu, and T. Honjo, unpublished results). We could not exclude a formal possibility that S-S rearrangement took place in a small portion of the sorted and tumor cells, and all the *trans*-mRNAs were produced by that portion of cells. However, this possibility seems very unlikely because most of the sorted cells carried human IgM and mouse IgG simultaneously on their surface and expressed the *trans*-mRNA together with transgenic human μ mRNA and sterile $C_\gamma 1$ transcripts, and the expression level of *trans*-mRNA in the lymphoma tumor was almost equal to that of the transgene transcript.

Obviously, our results do not exclude interchromosomal S-S recombination per se. In fact, other investigators suggested S-S recombination between transgenic μ and endogenous γ genes (44, 45). However, their cases differ from ours in that their transgenic mice contain a high-copy number of the murine μ gene as the transgene, and *trans*-expression was induced by specific antigen (arsenate) stimulation (44, 45). Because of the extensive homology between the endogenous loci and high-copy transgene, homologous recombination rather than S-S recombination might occur and a rare recombinational product could have been selected by antigen stimulation and during the hybridoma selection. The TG.SA transgene is shown to be integrated outside of the endogenous Ig H chain loci by breeding experiments (21). We have confirmed this by analyzing a hybridoma (2D9) that lacks the transgene but still retains the endogenous H chain locus of the TG.SA mouse (Fig. 6).

Discontinuous synthesis of mRNA is a feature of mRNA metabolism in several lower organisms (46), but has not been demonstrated in intact higher eukaryotic cells. The two well-characterized modes of discontinuous transcription are RNA-mediated priming or cap transfer (46) and *trans*-splicing (46–56). The former is known to occur in several viral systems, but this mechanism is probably not involved in the Ig *trans*-mRNA synthesis because joining at a splicing site like *trans*-mRNA does not occur by this mechanism and the transgenic human sequence has only partial homology with its murine counter part. On the other hand, *trans*-splicing was demonstrated in several lower eukaryotes (46–51), plant chloroplasts (52–54), and nuclear extracts of HeLa cell (55, 56). Although *trans*-splicing is a most likely and reasonable mechanism to generate the *trans*-mRNA, the following three unique features of the splicing donor make the present Ig *trans*-mRNA synthesis unprecedented. First, only specific pairs of transcripts are *trans*-spliced. No other *trans*-splicing systems require a specific splicing donor except for the self-catalyzed type in chloroplasts (57). Second, the Ig, the donor sequence of the *trans*-splicing, has a intron to be *cis*-spliced as the V region gene is composed of two exons. Third, the donor is part of intact transcription unit and thus processed by both *cis*- (for the μ mRNA) and *trans*- (for the second isotype mRNA) splicing. Therefore, novel molecular mechanism(s) should be involved in the Ig *trans*-mRNA synthesis by *trans*-splicing to determine the joining specificity of donor and acceptor combination, and to regulate the occurrence of *cis*- and *trans*-reactions at the same time. It should be of interest to see if such a mechanism(s) is confined in the immune system.

In so much as *trans*-splicing takes place in transgenic B cells stimulated with LPS and IL-4, it is reasonable to assume that the *trans*-splicing accounts for the Ig multiple isotype expression in B cells (12–15). This *trans*-splicing model is consistent with observations that the germline transcripts from specific C_H genes are expressed in association with class switching (16–19) and that F_1 rabbits express significant levels of V_H and C_H allotypic markers with *trans*-combination on membrane-bound and secreted Ig molecules (58–60).

Until now, the immunological significance of the cells that express multiple Ig isotypes has not been well established. One interesting possibility is that they might be intermediates for the class switching (2). Our data shown here would support this possibility because the second expressed isotype by

trans-splicing should correlate with the isotype of the germline transcripts and thus target specificity of S-S recombination. One of the most attractive yet speculative possibilities is that the second Ig isotype molecules expressed on the cell surface might produce a positive or negative feedback signal for S-S recombination by themselves or through the recognition and production of mediators by other type of cells. Thus, the B cell might be able to express the most suitable isotype for the specific circumstances quite efficiently in a large quantity. Such a stage of the cell would be transient and unstable, which might reflect the inability to establish stable cell lines that express multiple isotypes despite their existence in primary cultured cells (11, 13) or in vivo malignancies (15), and it might reflect the failure to keep the *trans*-mRNA expression of tumor no. 216 by transplantation into nude mice.

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