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Immunology

Immunoglobulin double-isotype expression by trans-mRNA in a human immunoglobulin transgenic mouse

(interleukin 4/two-color staining/polymerase chain reaction/nucleotide sequence/trans-splicing)

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ABSTRACT **We have studied immunoglobulin double-isotype expression in a transgenic mouse (TG.SA) in which expression of the endogenous immunoglobulin heavy chain locus is almost completely excluded by a nonallelic rearranged human μ transgene. By flow-cytometric analyses, we have shown that a small, but significant, portion (about 4%) of transgenic spleen cells expresses human μ (transgene) and mouse γ (endogenous) chains when cultured *in vitro* with bacterial lipopolysaccharide and interleukin 4. By using amplification of cDNA by the polymerase chain reaction, followed by cloning and sequencing of the amplified cDNA fragment, we have demonstrated expression of trans-mRNA consisting of the transgenic variable and endogenous constant (γ_1) region sequences. Such trans-mRNA could be produced by either switch recombination or trans-splicing between the transgene and endogenous sterile γ_1-gene transcripts. These results indicate that trans-splicing might be a possible mechanism for the immunoglobulin double-isotype expression in normal B lymphocytes that have not rearranged the second expressed constant region gene.**

During the course of B-lymphocyte differentiation, progeny of a single B lymphocyte can switch the expressed immunoglobulin isotype from IgM to IgG or other classes of immunoglobulin without changing the antigen specificity determined by the V region sequence (where V, D, J, and C indicate variable, diversity, joining, and constant regions of immunoglobulin). This phenomenon, known as immunoglobulin class switching, is accompanied by DNA rearrangement that takes place between S regions located 5′ to each C_H gene (except for the C_δ gene) (reviewed in refs. 1 and 2). The S–S recombination brings the V_HDJ_H exon to the proximity of the C_H gene to be expressed by deletion of other C_H genes located in-between on the same chromosome.

Although the deletion model has gained support by studies on myelomas, hybridomas, and normal spleen cells (3–6), the model has faced difficulty in explaining the observation that a single B lymphocyte carries on its surface more than one isotype (double or triple bearers) (7). Several groups analyzed sorted B cells or B-cell tumors carrying two isotypes and found no rearrangement of the second, expressed C_H gene, suggesting the involvement of RNA processing in double-isotype expression (refs. 8–11; T.-R.M., P. Sideras, A.S., and T.H., unpublished data). These findings led to the proposal that the double-isotype expression could be an intermediate stage to class switching (2, 8).

Interleukin 4 (IL-4), previously known as IgG1 induction factor, induces switching to IgG1 and IgE and simultaneously up-regulates transcripts (sterile transcripts) from the $C_{\gamma 1}$ and C_ε genes (12, 13). As the existence of such sterile transcripts from a C_H gene seemed to correlate well with switching to that particular C_H isotype, the sterile transcripts were thought to be indicative of opening of the chromatin structure of the C_H genes that make the putative switch recombinase system accessible (12–15).

The accessibility model (12–15) thus considered the sterile transcript useless by itself. However, there remains the possibility that the sterile transcript could be utilized as a substrate of the trans-splicing reaction for the double-isotype expression (P. Sideras, T.-R.M., A.S., and T.H., unpublished data). To test this hypothesis, we took advantage of a human immunoglobulin transgenic mouse (TG.SA) in which a majority of B lymphocytes expresses human transgenic μ chain and, by allelic exclusion, does not completely rearrange the endogenous immunoglobulin heavy chain locus (16). In this paper, we report that the transgenic V_HDJ_H exon can be expressed in conjunction with the endogenous mouse $C_{\gamma 1}$ gene. These results could accommodate either a trans-splicing or a DNA recombinant mechanism.¶

MATERIALS AND METHODS

Transgenic Mice. Two transgenic mouse lines carrying rearranged human V_HDJ_H-C_μ genes that allow expression of either the membrane-bound μ chain (TG.SA) or the secretory μ chain (TG.ST) were previously described (16, 17). These were maintained by crossing with FVB/N mice (Taconic Farms).

Spleen Cell Culture. Single cell suspensions were prepared by passing spleen cells through stainless steel mesh. Spleen cells, adjusted to concentrations of $\approx 3 \times 10^5$ per ml, were cultured in RPMI 1640 medium containing 50 μM 2-mercaptoethanol, 10% fetal calf serum, 30 μg of lipopolysaccharide (LPS) per ml (Sigma), and $\approx$100 units of recombinant mouse IL-4 per ml in 7% CO_2 in air at 37°C.

Flow Cytometry. Cells were washed twice with Hanks' balanced salt solution containing 0.01% sodium azide and 1% bovine serum albumin and preincubated with a mouse monoclonal antibody, 2.4G2 (18) (generous gift from Kevin Holmes, National Institutes of Health), to block the binding through the Fc receptors. Cells were then stained with fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-labeled goat polyclonal antibodies against the human or mouse μ or γ heavy chains (Southern Biotechnology Associates, Birmingham, AL). Cross-reactivities of these anti-

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Abbreviations: V, D, J, and C, variable, diversity, joining, and constant regions of immunoglobulin; LPS, lipopolysaccharide; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; IL-4, interleukin 4.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26532).

bodies were determined by staining normal mouse spleen cells and a cell line that expresses human IgM on its surface. There were no detectable cross-reactivities. Stained cells were analyzed by flow cytometry on a Cytofluorograf IIS (Ortho Diagnostics).

Polymerase Chain Reaction (PCR) and Characterization of Amplified DNA. Total cellular RNAs were extracted by the guanidium isothiocyanate method (19). mRNAs were converted to cDNA by using oligo(dT)$_{12-18}$ as primers and a cDNA synthesis kit (Boehringer Mannheim). These RNA/cDNA complexes were used as templates for the PCR. Approximately 5 ng of each cDNA was amplified by 25 cycles of PCR in a 50-μl reaction mixture (20) with 1.25 units of Taq DNA polymerase (*Thermus aquaticus* DNA polymerase) (Perkin-Elmer/Cetus) and then by another 25 cycles after the addition of the same amount of the enzyme using an automated DNA thermal cycler (Perkin-Elmer/Cetus). Primers for the PCR and DNA sequencing were synthesized by an automated DNA synthesizer (models 380A and 381A, Applied Biosystems). Primer sequences are indicated in the legend to Fig. 2. Amplified DNA fragments were analyzed on 2% low-melting agarose (LGT, FMC Bio Products) gel electrophoresis and recovered from the gel by heating to 65°C, extraction with a mixture of water-saturated phenol/chloroform/3-methylbutanol, 24:24:1, and precipitation with ethanol. The recovered fragment was subcloned into the *Sma* I site of Bluescript KS+ vector (Stratagene), and nucleotide sequences were determined by the dideoxy chain-termination method (21) using alkali-denatured plasmid DNA. The kit for DNA sequencing using 7-deazacytosine, restriction nucleases, T4 DNA ligase were purchased from Takara (Kyoto, Japan).

RESULTS

Double Bearers in Cultured Spleen Cells of the Transgenic Mouse TG.SA. As a first step we examined whether spleen cells from the transgenic mouse TG.SA could express murine γ and human μ chains simultaneously. Previous work with this transgenic strain has shown that a majority of splenic B lymphocytes expresses the transgenic human μ chain on their surface and that the endogenous immunoglobulin heavy chain locus is almost completely excluded (16). This observation was confirmed by two-color flow cytometry as shown in Fig. 1*a*. However, note that a small (1.5%), but significant, number of cells apparently express human and mouse μ chains on their surface. Few, if any, fresh cells from the TG.SA spleen expressed the transgene (μ) and endogenous mouse γ chain simultaneously (Fig. 1*b*).

As IL-4 is known to enhance isotype switching to IgG1 or IgE of LPS- or antigen-stimulated B cells (22, 23), we cultured the transgenic spleen cells with murine recombinant IL-4 and LPS. On day 4 of the culture when the proportion of the double bearers from normal mouse spleen cells reached a peak, we could detect a significant number of double bearers of the transgenic human μ and endogenous mouse γ chains (2.9% of live cells, Fig. 1*c*) among the spleen cells from the TG.SA mouse. A majority of these cells was not triple bearers of human μ, mouse μ, and mouse γ because only 0.9% (Fig. 1*d*) of the cells was positive for the mouse μ and γ chains.

When the cells were cultured longer (7 days) to the stage that most of the double bearers of the normal mice switch to γ single bearers, the population positive for human μ and mouse γ chains expanded in the transgenic spleen cells (4.1%, Fig. 1*e*) whereas that for mouse μ and γ chains did not (0.9%, Fig. 1*f*).

Detection of the Trans-mRNA Consisting of Transgenic Human V_HDJ_H and Endogenous Mouse C_γ Sequences by the PCR. Since the endogenous VDJ recombination is largely

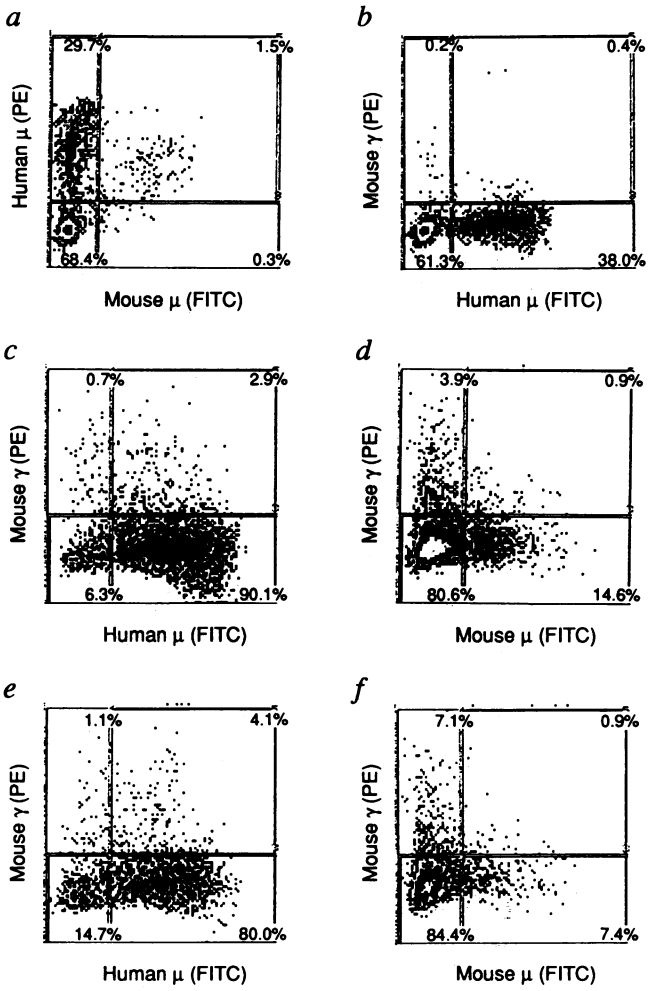


FIG. 1. Double-color flow-cytometric analyses of spleen cells from a transgenic mouse strain, TG.SA. Horizontal and vertical axes represent relative fluorescence of green (FITC) and orange (PE), respectively, in logarithmic scale. Spleen cells were stained with directly labeled antibodies against the human μ, mouse μ, or mouse γ chains. (*a* and *b*) Cells before the culture. (*c* and *d*) Cells cultured for 4 days with LPS and IL-4. (*e* and *f*) Cells cultured for 7 days with LPS and IL-4. Only live cells ($\approx 10^4$ cells in *a–d* and $\approx 5 \times 10^3$ cells in *e* and *f*) were analyzed. Background levels are indicated by lines. The percentage of cells in each region is shown at the corner.

excluded, the flow-cytometric analyses described above suggest that the mouse γ chain might be expressed together with the transgenic V_HDJ_H exon (Fig. 2*a*). To test this possibility, we extracted RNA from the cultured spleen cells of transgenic mice TG.SA and TG.ST and a human IgM$^+$ cell line (SA210) derived from TG.SA (recall that the TG.SA line expresses the membrane-bound form of human IgM and excludes endogenous immunoglobulin expression, whereas the TG.ST line expresses only the secreted form of human IgM and does *not* exclude). After converting the mRNAs into cDNAs by reverse transcriptase, we used PCR and amplified the postulated cDNA sequence that should have derived from the transgenic human V_HDJ_H-endogenous mouse $C_{\gamma 1}$ mRNA (trans-mRNA) (20, 24). Two pairs of primers were chosen so that the cDNA of the human transgene mRNA and trans-mRNA could be distinguished by the length as well as the sequence.

As shown in Fig. 2, the 397-bp fragment expected from the trans-mRNA was amplified only from TG.SA mRNA, whereas the 387-bp fragment from the transgenic μ gene was amplified from all three mRNAs. Because of the sensitivity of the PCR (20) we were very careful to avoid contamination

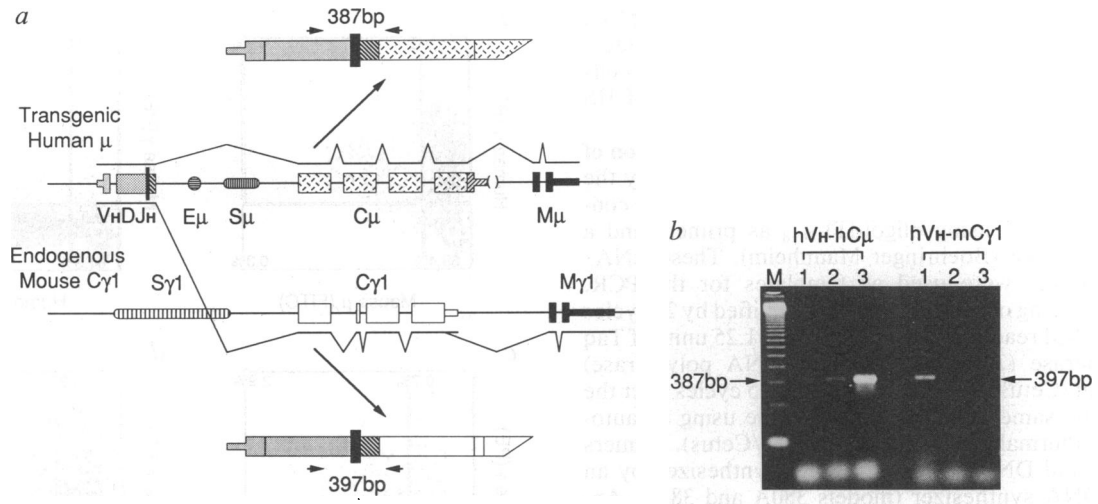


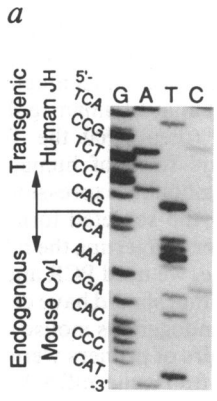
FIG. 2. Detection of the trans-mRNA by the PCR. (*a*) Schematic representations of the transgene, endogenous $C_{\gamma 1}$ gene, a part of transgenic μ chain mRNA, and a part of expected trans-mRNA. Exons are indicated as boxes with different fillings to distinguish functional regions. Enhancer and switch regions are also shown by circles and ellipses, respectively. Parentheses in the transgene indicate the deletion of the poly(A) addition site for the secretory μ chain. Expected sizes of PCR-amplified fragments with transgenic V_H primer (24-mer from 356 bases upstream to the end of the JH6 in the V_H sequence, sense strand) and human C_μ primer (31-mer from positions 31 to 1 of the CH1 exon, antisense strand) and with the transgenic V_H primer and mouse $C_{\gamma 1}$ primer (21-mer from positions 41 to 21 of the CH1 exon, antisense strand) are shown above and below the mRNA structures, respectively. bp, Base pairs. (*b*) Agarose gel electrophoresis of PCR-amplified DNA fragments. Amplified fragments (20 μl from 50-μl reaction mixture) of mRNA/cDNA from cultured spleen cells of TG.SA, TG.ST, and SA210 cells were applied to lanes 1, 2, and 3, respectively. Primer pairs used were human V_H/human C_μ (left) and human V_H/mouse $C_{\gamma 1}$ (right). A ladder of multiples of 123-bp fragments was used as a size marker (lane M). Sizes of detected fragments are shown.

of samples with each other or cloned DNAs, and we had never constructed artificial trans-mRNA or cDNA.

Structure of the Trans-mRNA Amplified by the PCR. To confirm the expected structure of the trans-mRNA and to exclude artifacts, the amplified 397-bp fragment was isolated by gel electrophoresis and subcloned into a plasmid vector for sequencing. Nucleotide sequences of three randomly chosen clones (clones 4, 5, and 12) were determined and compared with the transgene and mouse C_γ sequences. All three subclones had identical sequences except for the orientation in the vector. The sequences were identical to the transgenic V_HDJ_H sequence that was linked directly to the mouse $C_{\gamma 1}$ sequence at the precise exon–intron junction as shown in Fig. 3. The corresponding sequences of the transgenic and the amplified V_HDJ_H sequences are identical even at the junctions of the V_H, D, and J_H segments (N regions). These data unequivocally indicate that the trans-mRNA was derived from the human transgene and endogenous $C_{\gamma 1}$ gene (Fig. 3*b*).

DISCUSSION

Trans-Splicing or S–S Recombination in Transgenic B cells. By using flow-cytometric analyses we have shown that a significant number of TG.SA spleen cells expresses transgenic human μ and endogenous mouse γ chains when cultured with LPS and IL-4. Amplification by the PCR and cloning of the amplified fragment have unequivocally demonstrated expression of the trans-mRNA containing the transgenic V_HDJ_H and endogenous $C_{\gamma 1}$ sequences. Synthesis of this trans-mRNA could be due to either S–S recombination between the transgene and the endogenous $C_{\gamma 1}$ gene or trans-splicing (25–29) between the transgene transcript and the sterile transcript from the endogenous $C_{\gamma 1}$ gene. The exact location of the transgene is not known, although we



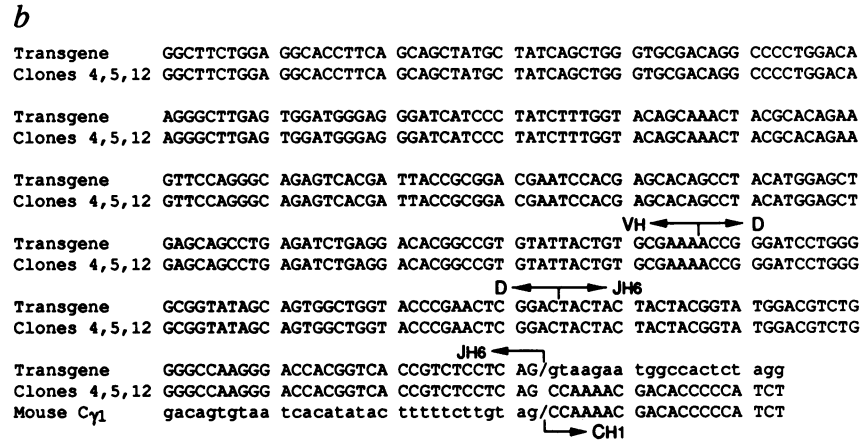


FIG. 3. Nucleotide sequence analysis of the amplified fragment of the trans-mRNA. (*a*) Auto-radiogram of sequence reading at the J–C exon junction. A reading of antisense strand of the exon junction of clone 4 is shown with the sequence converted to the sense strand at the left side. The junction is indicated by a horizontal line and arrows. (*b*) Nucleotide sequence of amplified fragment from the trans-mRNA. DNA sequence of clones 4, 5, and 12 (without the primer sequences used for the PCR) is shown with those of the transgene and mouse $C_{\gamma 1}$ gene. Junctions of the segments and exon–intron are shown by arrows and vertical lines.

know that the transgene is not located in the region empassing the J_H and C_H genes (M.C.N., unpublished data). Two copies of the transgene were integrated in a locus (unpublished data), and each transgenic mouse contained two copies of the transgene as the animal is heterozygous.

The combination of exons expressed in trans-mRNA does not seem random because we could not detect a trans-mRNA with the human V_H and the mouse μ sequences by the PCR (data not shown). This could be due to the requirement of specific sequences in the sterile transcript for trans-splicing or some specificity of DNA recombination. The fact that we could not detect trans-mRNA in the other transgenic mouse (TG.ST), which expresses only the secretory human μ chain and rearranges the endogenous immunoglobulin locus (17), could be due to a possible high efficiency of switch recombination between mouse endogenous genes and/or trans-splicing between their transcripts, excluding involvement of the transgene.

Durdik *et al.* (30) recently reported the expression of the V region sequence of the transgene in conjunction with the endogenous C_γ gene in hybridoma lines derived from hyperimmunized transgenic animals. The authors favor the DNA rearrangement model partly because of multiple copies of the transgenes. More recently, Sklar and his associates (31) reported trans-rearrangements between the γ and δ loci of the T-cell receptor genes.

Immunoglobulin Double-Isotype Expression in Normal B Cells. In view of the existence of trans-mRNA of the transgene and the endogenous $C_{\gamma 1}$ gene, we propose that trans-splicing could be a possible mechanism for double-isotype expression by B cells that have no DNA rearrangement of the second expressed C_H gene (refs. 8–11; T.-R.M., P. Sideras, A.S., and T.H., unpublished data). The trans-splicing model is consistent with the existence of the sterile transcripts from specific C_H genes (refs. 12–15; P. Sideras, T.-R.M., A.S., and T.H., unpublished data) and significant expression of trans-combination of V_H and C_H allotypic markers on B cells and immunoglobulin molecules in the F_1 rabbit (32–34). Trans-splicing and alternative splicing of a long transcript may not be mutually exclusive mechanisms. However, the long transcript model is short of explaining the precommitted isotype switching in pre-B-cell lines (12, 14, 15).

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