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Lineage-Specific Alternative Splicing of the Human FcγRIIA Transmembrane Exon Requires Sequences Near the 3′ Splice Site

M. A. KELLER,*[1] S. E. McKENZIE,† D. L. CASSEL,† E. F. RAPPAPORT,†
E. SCHWARTZ,*† AND S. SURREY*†

**Molecular Biology Graduate Group, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 and †Division of Hematology, The Children's Hospital of Philadelphia, Department of Pediatrics, The University of Pennsylvania School of Medicine, Philadelphia, PA 19104*

The human FcγRIIA gene produces multiple transcripts, including those with (FcγRIIa1) and without (FcγRIIa2) the single exon encoding the transmembrane domain (TM). Previously, a fluorescence-based RT-PCR assay showed lineage-specific differences in FcγRIIA transcript ratios (FcγRIIa2/FcγRIIa1). The mechanism of this lineage-specific expression was investigated in this study. Differential transcript stability does not play a major role, because transcript ratios remained constant in cells with both low (K562) and high (Dami) ratios following actinomycin D treatment. Transient expression studies in K562 and Dami cells using a minigene construct containing a 5.0 kb genomic fragment including the TM exon and adjacent intron and exon sequences showed recapitulation of endogenous transcript ratios. The TM exon was efficiently spliced in by the constitutive splicing machinery in HeLa cells, an FcγRIIA-negative cell line. Lineage-specific TM exon skipping was markedly diminished by two independent minigene mutations: a point mutation of the first nucleotide of the TM exon, and a five basepair intronic deletion near a putative branchpoint. These data demonstrate that *cis*-acting sequences in or near the TM exon 3′ splice acceptor site contribute to lineage-specific differences in FcγRIIA transcript ratios.

Fc receptors Soluble receptors Lineage specific Alternative splicing Minigene

THE humoral and cellular immune systems are linked by the binding of immunoglobulin to Fc receptors on the surface of effector cells (see Ravetch and Kinet, 1991, for review). Fcγ receptors bind IgG and are expressed by B and T lymphocytes, monocytes, macrophages, neutrophils, natural killer cells, megakaryocytes, and platelets (see van de Winkel and Capel, 1993, for review). There are three classes of Fcγ receptors in humans, FcγRI, II, and III. The FcγRII class is encoded by three highly homologous genes, FcγRIIA, FcγRIIB, and FcγRIIC (Brooks et al., 1989) and binds oligomeric IgG or immune complexes with a $K_a \geq 10^7\ M^{-1}$, but has negligible affinity for monomeric IgG (van de Winkel and Capel, 1993).

Multiple FcγRII transcripts are present in hematopoietic cells including FcγRIIa1, FcγRIIa2, FcγRIIb1, FcγRIIb2, FcγRIIb3, and FcγRIIc, where the lowercase letter (a, b, c) indicates the gene of origin (see van de Winkel and Capel, 1993, for review). Alternative splicing, resulting in distinct protein products from a single gene, is common in the FcγR gene family. For example, in the FcγRII class, FcγRIIb1 and FcγRIIb2 transcripts differ by alternative splicing of an intracytoplasmic exon and encode protein products with distinct functions (Tuijnman, et al., 1992; Amigorena, et al., 1992). In addition, the FcγRIIA gene produces the FcγRIIa2 transcript through alternative splicing of the exon encoding the single trans-

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[1]Address correspondence to Margaret A. Keller at her present address: DuPont Merck Pharmaceutical Co., Inc., Experimental Station E400-4245, P.O. Box 80400, Wilmington, DE 19880-0400.

membrane (TM) domain, which results in a protein product that can be secreted from the cell (Astier et al., 1993).

Soluble FcγRIIa2 receptors have been detected in the conditioned medium of human hematopoietic cell lines (Rappaport et al., 1993), cultured human epidermal Langerhans cells (de la Salle et al., 1992), as well as in human saliva (Sautes et al., 1992) and serum (Astier et al., 1993). Soluble Fcγ receptor competitively inhibits binding of antibody-antigen complexes to the membrane form of the receptor, thereby downregulating integral membrane receptor signaling (Sautes et al., 1990). Inhibition of the reverse passive Arthus reaction in rats by soluble recombinant FcγRIIa2 supports a role for soluble Fcγ receptors in regulation of the immune response (Sautes et al., 1992). Understanding how the production of such a regulatory molecule is controlled will aid in the understanding of immune function and perhaps lead to innovative therapeutic regimens for immunologic diseases.

Alternative splicing of exons encoding single transmembrane domains is a widespread phenomenon in many receptor families in hematopoietic cells (CD8, Giblin et al., 1989; HLA-DQβ, Briata et al., 1989). Little is known about the regulation of alternative splicing in these cells. Recently, analysis of the FcγRII transcripts in hematopoietic cell lineages using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Keller et al., 1993) demonstrated that expression of the FcγRIIA transcripts is regulated in a lineage-specific manner. In this work, we utilize this method to examine the mechanism of regulation of the ratios of alternatively spliced FcγRIIA transcripts and delineate *cis*-acting elements involved in this regulation.

MATERIALS AND METHODS

Mammalian Cells and Cell Lines

HEL, U937, Jurkat, Raji, RPMI 1788, K562, and HeLa cell lines were obtained from the American Type Culture Collection (Rockville, MD), and CHRF-288-11 and Dami cells were obtained from Drs. D. Witte (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, Cincinnati, OH) and S. Greenberg (Department of Medicine, Harvard Medical School, Boston, MA), respectively. Cell lines were maintained in RPMI 1640 (Cellgro, Fisher Scientific, Pittsburgh, PA) supplemented with 10% (v/v) fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, and 100 U/ml each of penicillin and streptomycin, all obtained from Gibco BRL (Gaithersburg, MD). K562 cells were also maintained in Macrophage SFM Medium (Gibco BRL) supplemented with L-glutamine, penicillin, and streptomycin, whereas Dami cells were maintained in Iscove's Modified Dulbecco's Medium (Cellgro) supplemented with 10% (v/v) horse serum (Sigma), L-glutamine, penicillin, and streptomycin. All lines were grown at 37°C with 5% CO_2. In some cases, Dami and K562 cells (0.5×10^6 cells/ml) were grown in medium supplemented with actinomycin D (10 μg/ml) (Sigma) for 20 min to 8 h.

Human platelets were isolated from platelet-rich plasma obtained by differential centrifugation of whole blood from normal donors (white cell to platelet ratio, 1 : 1000). Human neutrophils were isolated by centrifugation through Lymphocyte Separation Medium (Organon Teknika Corp., Durham, NC) and human monocytes by adherence to gelatin-coated flasks as described previously (McKenzie et al., 1993).

Mammalian Cell Transfections

HeLa cells were transfected by lipofection using DOTAP (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's protocol. Dami and K562 cells were electroporated in sterile electroporation chambers (Gibco BRL) at 200 V and 800 μF in a Cell-Porator (Gibco BRL) using 30 μg test plasmid and 30 μg HS432γ1.3pXP1 control plasmid per 10^7 cells. Cells were grown for 24 h posttransfection.

Luciferase assays were performed to analyze transfection efficiency. Cells were lysed in 100 μl Triton Lysis Buffer [1% (v/v) Triton X-100, 50 mM Tris (pH 7.6), 1 mM DTT] and lysates were assayed for luciferase activity as follows: 5, 10, and 20 μl of lysate were assayed in 10 mM Tris (pH 7.8), 15 mM $MgSO_4$, 2 mM ATP (Sigma), and 0.2 mM D-luciferin, potassium salt (Analytical Luminescence Laboratory, San Diego, CA). Relative light units produced were measured in a Lumat luminometer (Berthold, Pittsburgh, PA), and data were analyzed for linearity of lysate volume vs. relative light units using Cricket Graph III (Cricket Software, Malvern, PA).

RNA Extraction

Cells were harvested and total RNA isolated using RNAzol B or RNA Stat-60 (Tel-test Inc., Friendswood, TX). RNA concentrations were de-

termined spectrophotometrically and quality was assessed by nondenaturing gel electrophoresis.

Northern Blot Analysis

Northern blot analyses were performed using standard procedures (Sambrook et al., 1989). Gels were transferred to Zetabind membranes (Cuno, Meriden, CT) overnight in 1× High Efficiency Transfer Buffer (Tel-test) by capillary action. Membranes were air dried, UV cross-linked in a Stratalinker (Stratagene, La Jolla, CA) followed by prehybridization and hybridization in Rapid Hybridization Buffer (Amersham, Arlington Heights, IL) following the manufacturer's protocol in a Hybaid hybridization oven (National Labnet Co., Woodbridge, NJ). Blots were washed and exposed to X-AR film (Eastman Kodak, Rochester, NY). Hybridization probes, which included human β_2-microglobulin cDNA, kindly provided by Dr. M. Kiledjian (University of Pennsylvania, Philadelphia, PA) and human *c-myc* cDNA by Dr. H. Nyugen (University of Pennsylvania) were labeled using the Ready-To-Go Labeling kit (Pharmacia, Piscataway, NJ) and unincorporated nucleotides were removed with Midi-Select D G-50 spin columns (5′-3′, Boulder, CO).

Oligonucleotide Primers

Primers were synthesized by the Nucleic Acid/Protein Core facility at the Children's Hospital of Philadelphia, with the exception of the dye-tagged 3′ primer 224M-F (where F represents the fluorescein molecule attached to the 5′ end of the oligonucleotide 224M), which was synthesized by Research Genetics, Inc. (Huntsville, AL). The primers HFc1S and 224M used to specifically amplify the FcγRIIA transcripts have been described previously (Rappaport et al., 1993; Cassel et al., 1993). Oligonucleotide sequences are shown below with point mutation (underlined) and site of deletion (asterisk) indicated:

HFc1S	5′-TCA CTA TAG GGA GAC CCA AG-3′
224M	5′-GCT CAA ATT GGG CAG CCT TCA-3′
IFOL-1	5′-AGC TGG GAT GAC TAT GGA A-3′
IFOL-2	5′-AGC TTT CCA TAG TCA TCC C-3′
V2S	5′-TCA CTA TAG GGA GAC CCA AG-3′
MUT 5′SSA	5′-ATG CTG GGC GCT AGG GAA GA-3′
TMBPA	5′-CAG GAT GGG GGG TTA CTA TTT TAA TG-3′
HFc2BC1M1	5′-GTT TCT CAG GGA GGG TCT CT-3′
TTMCS	5′-CTG TCT TCC CTA GCC CCC AG-3′
TMBPS	5′-GTA ACC CCC CAT CCT G*AT GTC TGT CTT CCC TAG-3′

Fluorescence-Based RT-PCR

The fluorescence-based RT-PCR assay was performed as described previously (Keller et al., 1993) utilizing one PCR primer that was tagged with a fluorescein residue at the 5′ end. This allowed the PCR product to be measured through fluorescence detection. Total input of 2.5 μg RNA and 25 PCR cycles were the experimental parameters that were shown to result in output fluorescence intensity in the linear range of amplification in this system (Keller et al., 1993). When endogenous FcγRIIA transcript ratios were analyzed, the 5′ primer was HFc1S; when exogenous, minigene-derived transcript ratios were analyzed, the 5′ primer was V2S, complementary to sequences derived from the vector and present in the 5′ end of the minigene-derived RNA. The 3′ primer in each case was 224M-F. Detection and quantitation of fluorescence-based RT-PCR reactions was performed as described previously (Keller et al., 1993). Briefly, 1 μl of each PCR product was electrophoresed on polyacrylamide gels [endogenous transcripts were analyzed on 4% or 5% (w/v) polyacrylamide gels and RT-PCR products from minigene transfections on 6% (w/v) polyacrylamide gels] using a 373A DNA Sequencer with photomultiplier and the data were analyzed using Gene Scanner software GS Analysis, both from Applied Biosystems (Foster City, CA).

Plasmid Construction

An FcγRIIA minigene was created by subcloning a 5.0-kb Bam HI fragment from the genomic clone λFc8.1 (McKenzie et al., 1992), beginning within the second extracellular (EC2) exon and ending in the intron 3′ to the first cytoplasmic (C1) exon, into the unique Bam HI site in the polylinker of pcDNA1/Amp vector (Invitrogen, San Diego, CA). The vector supplies a cytomegalovirus promoter (pCMV) and SV40 small t intron and polyadenylation signals. In some systems, translatability influences transcript stability (see Jackson, 1993, for review); therefore, an approximately 20-bp double-stranded fragment containing the FcγRIIA translation start site was engineered into the construct by annealing two

overlapping oligonucleotides (IFOL-1 and IFOL-2). The resulting wild-type minigene was called p5.0AUG (see Fig. 3A).

The control plasmid, HS432γ1.3pXP1, used to assess transfection efficiency (kindly provided by R. Emery and C. Stoeckert of Children's Hospital of Philadelphia), contained sequences from the human globin Locus Control Region inserted upstream of 1.3 kb γ-globin gene promoter inserted 5′ of the luciferase cDNA in pXP1 (de Wet et al., 1987). This plasmid confers high levels of luciferase expression in the cell lines used in these studies.

Subcloning of plasmid constructs was performed using standard techniques (Sambrook et al., 1989). Bacterial transformation was performed using competent DH5α cells (Gibco BRL). All junctions created by subcloning were subjected to DNA sequence analysis. Large-scale preparations of plasmid DNA were performed by alkaline lysis using Qiagen tip-500 column purification (Qiagen, Inc., Chatsworth, CA).

Site-Directed Mutagenesis of Minigenes

Overlap extension PCR (Ho et al., 1989) was used to create mutations in the FcγRIIA minigene using Vent DNA Polymerase (New England BioLabs, Beverly, MA). The primer pairs V2S/Mut-5′SSA and TTMCS/HFc2BC1M1 were used to amplify PCR fragments that contained a T to C point mutation at the overlapping ends. These fragments were combined in a second PCR reaction using the outer primers V2S and HFc2BC1M1 and the resulting product was subcloned into p5.0AUG, replacing the analogous sequence in the wild-type minigene, and creating p5.0AUG-TC6. p5.0AUG-Δ5-4, which contains a 5 bp deletion in the EC2-TM intron, was created with PCR reactions that used the initial pairs V2S/TMBPA and TMBPS/HFc2BC1M1 and the second pair V2S/HFc2BC1M1. The sequences of p5.0AUG-TC6 and p5.0AUG-Δ5-4 clones were analyzed over the entire area amplified by PCR, and were free of unwanted PCR-induced mutations.

DNA Sequence Analysis

PCR cycle sequencing was performed using the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Excess terminators were removed using Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ) and products were analyzed on a 373A DNA Sequencer (Applied Biosystems) by the Nucleic Acid/Protein Core Facility at the Children's Hospital of Philadelphia.

RESULTS

Determination of FcγRIIA Transcript Ratios

Transcript ratios for FcγRIIa2/FcγRIIa1 mRNA were determined using a fluorescence-based, quantitative RT-PCR assay (Keller et al., 1993). Briefly, differently sized (±123 bp TM exon), fluorescently tagged PCR products for the two species are separated by electrophoresis, and quantitative assessment of fluorescence intensity in each peak is evaluated and ratios determined. Fluorescence output and the resulting transcript ratios are shown in Table 1 using mRNA from Dami cells as an example.

The ratios of alternatively spliced FcγRIIA transcripts in a variety of cells are presented in Fig. 1. Among primary cells, human platelets had

TABLE 1
FLUORESCENCE-BASED RT-PCR DATA FOR RNA FROM Dami CELLS

Experiment	FcγRIIa2 (TM^-)	FcγRIIa1 (TM^+)	Transcript Ratio
1	5,053	9,568	0.53
2	7,829	15,205	0.51
3	5,943	13,198	0.45
4	2,541	6,194	0.41
5	2,488	6,395	0.39
6	4,003	10,297	0.39
7	2,598	6,002	0.43

The values shown in columns 2 and 3 are the fluorescence intensities for the appropriate bands on the polyacrylamide gel generated by the Gene Scanner, and were used to calculate the transcript ratios (FcγRIIa2/FcγRIIa1) shown in column 4. These data were used to calculate the average ratio of 0.44 ± 0.05 shown as a histogram in Fig. 1. Similar calculations were used for all transcript ratio calculations.

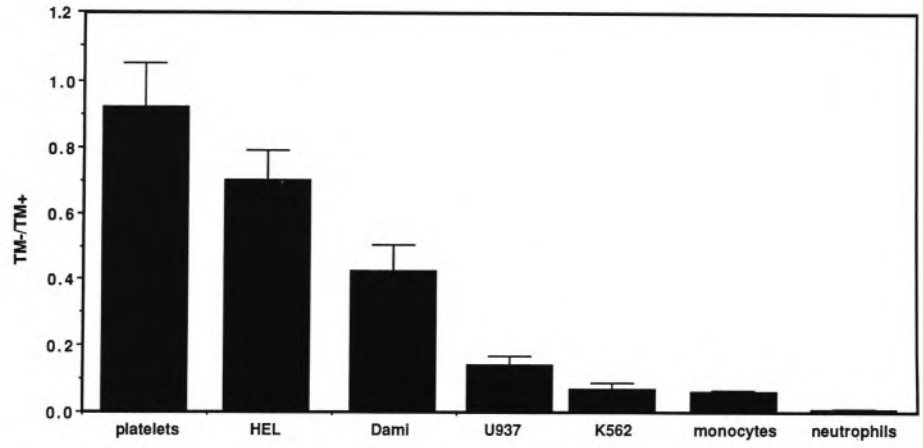

FIG. 1. FcγRIIa2/FcγRIIa1 transcript ratios in hematopoietic cells and cell lines. TM^-/TM^+ (FcγRIIa2/FcγRIIa1) transcript ratios are shown (mean ± SD of three to five independent experiments for each primary cell or cell line). Data for platelets, Dami cells, monocytes, and neutrophils are compared with that presented previously for HEL, K562, and U937 cells (Keller et al., 1993).

the highest FcγRIIa2/FcγRIIa1 transcript ratio, 0.91 ± 0.16 (mean ± SD); monocytes had a ratio of 0.06 ± 0.01 and neutrophils had no detectable FcγRIIa2 in the presence of an abundance of FcγRIIa1. The human erythroleukemic cell line, HEL, is bipotential, having both erythroid and megakaryocytic properties (Papayannopoulou et al., 1983). HEL and the megakaryoblastic cell line, Dami, were used as models of megakaryocyte gene expression and had the highest soluble/membrane transcript ratios (0.70 ± 0.09 and 0.44 ± 0.05, respectively), whereas the multipotential cell line, K562 (0.07 ± 0.02), and the monocytic cell line, U937 (0.04 ± 0.03), expressed much lower ratios. These results confirm that FcγRIIA transcript ratios are regulated in a lineage-specific manner.

Examination of Transcript Stability

Lineage-specific differences in transcript ratios could arise from differences in stability of the FcγRIIa2 and FcγRIIa1 messages in various lineages. K562 (low FcγRIIa2/FcγRIIa1) and Dami (high FcγRIIa2/FcγRIIa1) cells were treated with actinomycin D over an 8-h period, spanning the known 3-h half-life of FcγRIIA transcripts (Comber et al., 1992) and the soluble/

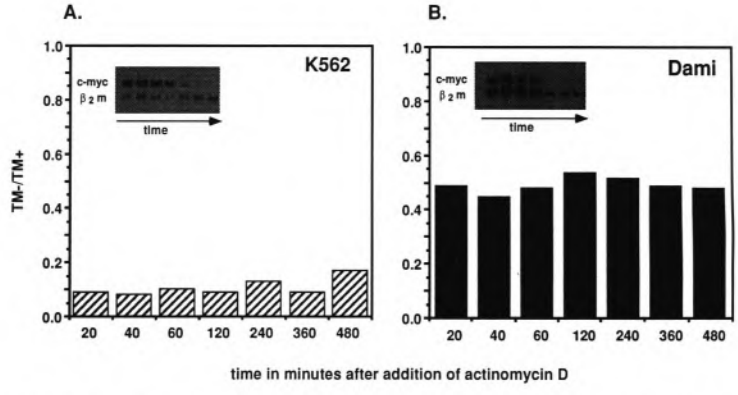

FIG. 2. FcγRIIA transcript ratios in actinomycin D-treated cell lines. (A) TM^-/TM^+ (FcγRIIa2/FcγRIIa1) transcript ratios at indicated times after addition of actinomycin D to K562 cells are shown. Inset shows Northern blots prepared with the same RNA samples used in the ratio determinations hybridized to radiolabeled human *c-myc* and β_2-microglobulin probes. (B) Same as (A) using Dami cells.

membrane-bound transcript ratios determined at various times after actinomycin D addition (Fig. 2). Northern blot analysis was performed using human *c-myc* and β_2-microglobulin cDNA probes (Fig. 2, insets). Transcription was abrogated by actinomycin D treatment as expected: the short half-life *c-myc* message was undetectable after 2 h whereas the long half-life β_2-microglobulin message was unaffected. Transcript ratios were unaffected by actinomycin D treatment (Fig. 2), indicating that differential transcript stability is not the cause of lineage-specific differences in transcript ratios. These data imply that transcript ratio differences are due to regulation at the level of alternative splicing.

Examination of Alternative Splicing

A minigene assay system was designed to decipher the role of alternative splicing in the lineage-specific regulation of FcγRIIA and to investigate *cis*-acting elements that might play a role in this regulation. The minigene containing the TM exon and surrounding exons (p5.0AUG, Fig. 3A) was transfected into HeLa cells to assess how the minigene-derived pre-mRNA would be processed by cells that do not express FcγRIIA. As shown in Fig. 3B, HeLa cells transfected with the minigene gave rise to a transcript ratio of 0.04 ± 0.01. The low ratio demonstrates that the "default" processing pathway is inclusion of the TM exon, with greater than 95% of transcripts containing the TM exon in these cells.

Dami cells transfected with the wild-type minigene had a transcript ratio of 0.41 ± 0.03 and K562 cells had a transcript ratio of 0.11 ± 0.01 (Fig. 3B). Comparison of these data with the endogenous transcript ratios in these hematopoietic cells (Figs. 1 and 3B) demonstrates that the 5.0-kb genomic fragment in the minigene construct is sufficient to recapitulate in vivo regulation of alternative splicing and, thus, must include the *cis*-acting elements required for lineage-specific differences in transcript ratios.

Minigenes were constructed with either a point mutation at the first nucleotide (T) of the TM exon or a deletion of a 5 nt element near the 3′ splice site (Fig. 4). These sequences were targeted because of their divergence from FcγRIIB and FcγRIIC, which do not splice out the TM exon. The point mutation (T to C) was made in the wild-type minigene, p5.0AUG, creating p5.0AUG-TC6; the 5 nt element was deleted in p5.0AUG, creating p5.0AUG-Δ5-4. These mutant minigenes were transfected into Dami and K562 cells as well as

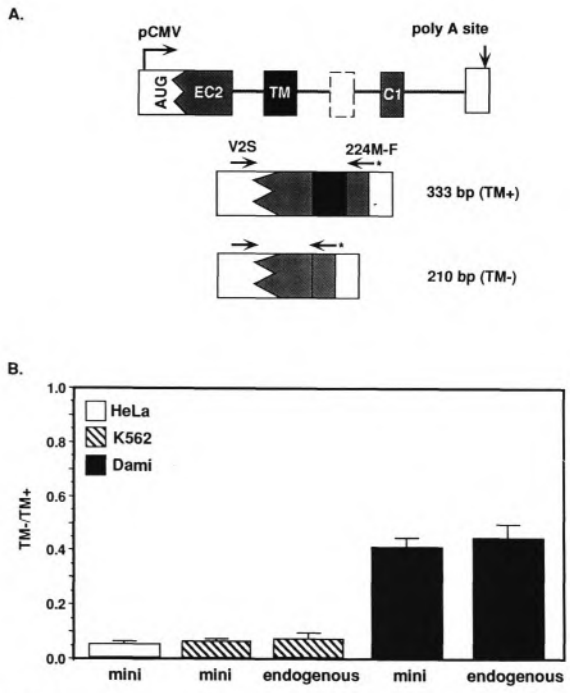

FIG. 3. FcγRIIA minigene structure and minigene-derived transcript ratios in HeLa, Dami, and K562 cells. (A) Schematic diagram of minigene construct, p5.0AUG (top line), containing EC2, TM, ψC pseudoexon (Qui et al., 1990, box with dashed outline), C1 exon and intron sequences (solid horizontal lines). Fluorescence-based RT-PCR was performed on RNA harvested from transiently transfected cell lines using a vector-derived sense primer (V2S) located in the 5′ end of the message and a fluorescently tagged (*) antisense primer (224M-F) located in the C1 exon. Products were 333 or 210 bp in length for TM^+ and TM^- transcripts, respectively. (B) TM^-/TM^+ (FcγRIIa2/FcγRIIa1) transcript ratios were determined for HeLa, K562, and Dami cells with each bar representing the mean of at least three separate transfections with standard deviations shown. The labels "mini" and "endogenous" refer to transcript ratios derived from minigene-transfected cells using the minigene-specific primer pair (V2S/224M-F), or from untransfected cells using the endogenous primer pair (1S/224M-F).

into HeLa cells. Minigene-derived transcript ratios were calculated from at least three different transfections (Fig. 5). Although the wild-type minigene gave a ratio of 0.41 ± 0.03 in Dami cells, the T to C mutation resulted in a transcript ratio of 0.06 ± 0.01 (Fig. 5). The transcript ratio in K562 cells decreased from 0.11 ± 0.01 to 0.06 ± 0.02. Thus, the transcript ratio in Dami and K562 cells transfected with the minigene containing the T to C mutation is the same (0.06). These data demonstrate that lineage-specific alternative splicing of the TM exon is eliminated by introduction of a point mutation at the first nucleotide of the TM exon.

The deletion of the 5 nt element also had a significant effect on transcript ratios. Dami cells

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hFcγRIIA  gtgggacagggagaatacaaacgttgtcattaaaatagtaacccccc
hFcγRIIB  ********a*****g***------**------------------**t
hFcγRIIC  ********a*****g***------**------------------**t

hFcγRIIA  atcctgccctaatgtctgtcttccctagTGCCCAGCATGGGCAGCTC
hFcγRIIB  g*****-----a***************CT******---------**
hFcγRIIC  g*****-----a***************CT******---------**

hFcγRIIA  TTCACCAATGGGGATCATTGTGGCTGTGGTCATTGCGACTGCTGTAG
hFcγRIIB  ******G************************C**G**T********
hFcγRIIC  ******G************************C**G**T********

hFcγRIIA  CAGCCATTGTTGCTGCTGTAGTGGCCTTGATCTACTGCAGGAAAAAG
hFcγRIIB  *G*******************************************
hFcγRIIC  *G*******************************************

hFcγRIIA  CGGATTTCAGgtttgtagctcctccc
hFcγRIIB  *************************
hFcγRIIC  *************************
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FIG. 4. Sequence comparison of human FcγRIIA, B, and C genes. TM exon and surrounding sequences are aligned for human (h) FcγRIIA hFcγRIIB, and hFcγRIIC genes (GenBank accession numbers L08107, L08108, L08109, respectively). Asterisks indicate sequence identity and dashes represent gaps; sequences different from FcγRIIA are shown below. Uppercase and lowercase letters are exonic and intronic sequences, respectively. The T, 5 nt, and 9 nt elements discussed in the Results and Discussion are underlined, and the putative branch-point adenosine located at −17 nt from the 3′ splice site is depicted in bold.

transfected with the wild-type minigene have a transcript ratio of 0.41 ± 0.01 whereas the ratio resulting from transfection with the deletion construct (p5.0AUG-Δ5-4) decreased to 0.12 ± 0.02. The transcript ratio of K562 cells also decreased, from 0.11 ± 0.01 to 0.05 ± 0.02. The ratio in HeLa cells was not significantly altered by either mutation (from 0.04 ± 0.01 to 0.01 ± 0.01 with p5.0AUG-TC6, and from 0.02 ± 0 to 0.04 ± 0.01 with p5.0AUG-Δ5-4). These data demonstrate that the first nucleotide of the TM exon, as well as the five nucleotides in the EC2/TM intron, are necessary for the appropriate lineage-specific alternative splicing of FcγRIIA.

DISCUSSION

Our previous work demonstrated that expression of the alternatively spliced transcripts, FcγRIIa1 and FcγRIIa2, was regulated in a lineage-specific manner in hematopoietic cell lines (Keller et al., 1993). That work is extended here, showing that megakaryocytic cells (platelets) and cell lines with megakaryocytic properties (HEL and Dami) showed the highest transcript ratios (FcγRIIa2/FcγRIIa1). These results suggest that platelets may be a major source of the soluble protein. The facts that HEL cells express the soluble protein (Rappaport et al., 1993) and that platelets can be

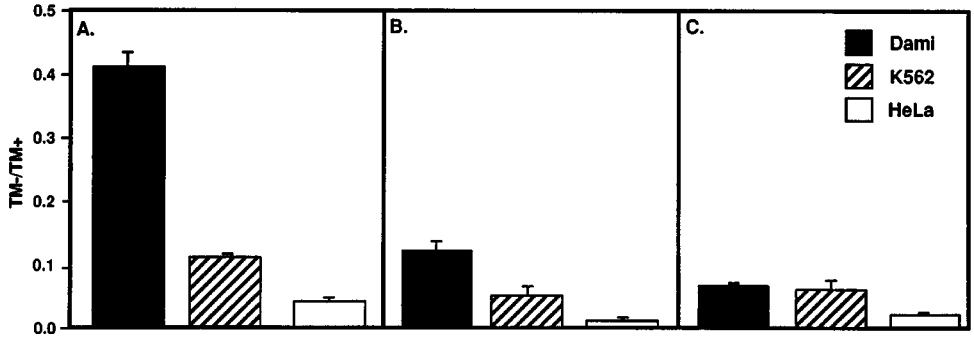

FIG. 5. Effects of mutations near the 3′ splice site on minigene-derived transcript ratios. TM^-/TM^+ (FcγRIIa2/FcγRIIa1) transcript ratios are shown for Dami (black bars), K562 (striped bars), and HeLa (white bars) cells transfected with (A) wild-type (p5.0AUG), (B) T to C (p5.0AUG-TC6), and (C) 5 nt deletion (p5.0AUG-Δ5-4) FcγRIIA minigene constructs.

stimulated to release this protein (Fridman et al., 1993) support this hypothesis. Further studies are now required to delineate the physiological function of this soluble receptor.

Our data demonstrate that lineage-specific expression of FcγRIIA mRNA is regulated at the level of alternative splicing. First, the FcγRIIa2/FcγRIIa1 transcript ratio did not change with actinomycin D treatment, indicating that differential transcript stability does not play a major role. Second, a 5.0-kb fragment of FcγRIIA genomic DNA containing the alternatively spliced TM exon recapitulated the endogenous transcript ratios in a transient expression system. Third, mutations in or near the 3′ splice site of the TM exon significantly altered transcript ratios. Taken together, these data strongly support a model of alternative splicing in which sequences in or around the 3′ splice site play a role in lineage-specific regulation.

Sequences surrounding the TM exon in FcγRIIA as well as in the closely related human FcγRIIB and FcγRIIC genes were compared (Fig. 4). FcγRIIA undergoes alternative splicing of the transmembrane exon (Rappaport et al., 1993) whereas FcγRIIB and FcγRIIC do not (Cassel et al., 1993). The 5′ splice sites are identical in the three human genes (Fig. 4), suggesting that this area is not responsible for the TM exon skipping seen in FcγRIIA. Several areas were divergent, however (Fig. 4), and these sequences were candidates for mutagenesis.

First, FcγRIIA differs from the others at position +1 of the 3′ splice site (the first nucleotide of the TM exon) (Fig. 4). The base is a T in FcγRIIA and a C in FcγRIIB and FcγRIIC. The most common nucleotide at this position in mammals is a G (51%), followed by C (30%), A (13%), and T (6%) (Shapiro and Senapathy, 1987). In our studies, when this nucleotide was changed from a T to a C in FcγRIIA, splicing out of the TM exon was greatly diminished (Fig. 5). Thus, in human FcγRIIA, the presence of a T at TM splice acceptor site position +1 is crucial for TM exon skipping. Second, there is a putative branch adenosine at −17 in the three human genes, but the surrounding sequences in FcγRIIB and FcγRIIC are distinct from that in FcγRIIA (Fig. 4). There are five additional basepairs in FcγRIIA that are not present in FcγRIIB and FcγRIIC in this region. Deletion of these five bases in the FcγRIIA minigene construct created a putative branch identical to those of FcγRIIB and FcγRIIC, and resulted in decreased TM exon skipping (Fig. 5). Together, these data demonstrate that both the T element and the five-nucleotide element are involved in regulating TM exon inclusion and may affect TM splicing by altering branchpoint selection, secondary structure, and/or interaction with a *trans*-acting factor(s).

RNA secondary structure has been shown in other systems to affect 3′ splice site (Deshler and Rossi, 1991) as well as branchpoint selection (Chebli et al., 1989; Goguel et al., 1993). The sequences surrounding the FcγRIIA TM 3′ splice site may be involved in basepairing that could affect the accessibility of the RNA branchpoint to splicing factors, such as U2 snRNA (Wu and Manley, 1989). This basepairing might involve the 5 nt element as well as a 9 nt element present in the TM exon of FcγRIIA, but not found in FcγRIIB or FcγRIIC (see Fig. 4). Exonic elements or exon recognition sequences (ERS) have been shown to play a role in the regulation of alternative splicing, acting as binding sites for protein factors (Kister et al., 1993; Watabake et al., 1993). Delineation of the role of *trans*-acting factors in TM exon inclusion will require studies of RNA–protein interactions.

Based on the data presented here, a model of regulated FcγRIIA alternative splicing can be proposed. A cell that produces significant amounts of the FcγRIIa2 transcript might contain a factor that requires the T element and the 5 nt element to exert its optimal effect (i.e., blockage of TM exon inclusion). Binding of factor could stabilize message secondary structure and result in inaccessibility of the branchpoint to binding by U2 RNA, an essential step in spliceosome assembly (see Wise, 1993, for review). This model predicts that Dami cells, which have a high FcγRIIa2/FcγRIIa1 transcript ratio, would possess such a factor and that this factor would be present in lesser amounts or in an inactive state in HeLa and K562 cells. The activity of some splicing factors is altered by their state of phosphorylation (Mermoud et al., 1992; Tazi et al., 1992) as well as by their amounts relative to other splicing factors (Fu et al., 1992; Mayeda and Krainer, 1992; Mayeda et al., 1993).

The present study has identified *cis*-acting elements involved in the regulated alternative splicing of the transmembrane exon of human FcγRIIA. Identifying the *trans*-acting factors that regulate FcγRIIA TM exon skipping may lead to new insights into regulation of gene expression in hematopoietic cells.

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